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Letters to the Editors

THE AMINO ACID REQUIREMENTS OF TWENTY-THREE LACTIC ACID BACTERIA*

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Microbiological procedures which are convenient and readily adaptable to routine assays have been described for nearly all of the commonly occurring amino acids. Although six strains of lactic acid bacteria[1] have been employed in the determination of amino acids, it seemed desirable to study the nutritional requirements of other organisms and, if possible, to make available additional assay procedures. Twenty-three strains of lactic acid bacteria have been investigated in the present work.

The amino acid requirements of numerous strains of lactic acid bacteria were studied by Orla-Jensen *et al.* (2) in 1936. Since that date, there have been comparable investigations of *Leuconostoc mesenteroides* P-60 (3), *Streptococcus salivarius* (4), *Streptococcus faecalis* R (4–6), *Lactobacillus plantarum* (7), *L. buchneri* (8), *L. mannitopoeus* (8), *L. lycopersici* (8), *L. delbrückii* LD5 (9), *L. arabinosus* 17-5 (10–12), *L. casei* ε (13), and *L. fermenti* 36 (14).

In the present work, it was desired to determine the response when the lactic acid bacteria were cultured on a basal medium containing the types and concentrations of nutrients which would promote rapid and abundant acid production. It was anticipated from earlier experiences that the requirements of an organism for a given nutrient, established under such conditions, would not disappear or change in the presence of substances introduced during the assay of a test material. It has been found generally true that microbiological methods which depend for their specificity on basal media which lack metabolites, or have suboptimal concentrations of essential factors, are likely to be unreliable and of limited utility.

[1] *Leuconostoc mesenteroides* P-60, *Streptococcus faecalis* R, and the lactobacilli *arabinosus* 17-5, *casei* ε, *delbruckii* LD5, and *fermenti* 36.

EXPERIMENTAL

The response of each organism listed in Table II, at 35°[2] and from 5 to 7 incubation times, on the complete basal medium given in Table I, and on the same medium with each of the amino acids omitted in turn, was measured by titrating the combined solutions from duplicate tubes with 0.020 to 0.035 N NaOH in the presence of about 0.7 mg. of brom-thymol blue indicator. The total volume of medium per tube was 1 ml. per 3 inch test-tube or 1.5 ml. per 4 inch test-tube. The inocula were prepared by growing the organism 24 hours on the medium given in Table I, except that an acid hydrolysate of 7.5 gm. of casein was substituted for all of the amino acids other than cysteine, norleucine, and tryptophane. The cells were centrifuged and resuspended in a volume of sterile saline equal to 3 times that of the medium in which they were grown. Each tube was inoculated with approximately 0.1 ml. of this cell suspension. The basal medium and the inoculum were transferred to the tubes with the aid of the Brewer automatic pipette (Baltimore Biological Company). Prior to inoculation the tubes of medium were autoclaved for 5 minutes at 15 pounds in racks each containing 330 tubes covered with a closely fitting cap made from three layers of toweling. After tubes were removed from the incubator, growth was arrested by steaming the rack of tubes for 15 minutes at 100°.

The experimental results are shown in Tables II to IX.

DISCUSSION

The composition of the basal medium employed in the present experiments is given in Table I. It was expected that this medium would be reasonably satisfactory, since it contained four constituents (choline, inositol, pyridoxamine, and pyridoxal) in addition to those present in media used previously for the quantitative determination of a number of amino acids. A further possible improvement in nutritional quality was effected by increasing several-fold the concentrations of total vitamins and amino acids.

That this medium is not fully optimal for all strains of lactic acid bacteria is evident, since it did not permit any growth of *Lactobacillus acidophilus* (4357) or of *Lactobacillus plantarum* (8008). As shown in Table II, the rate and amount of acid production over incubation periods ranging from 6 up to 143 hours were relatively high only for *Leuconostoc citrovorum* (8081), *Leuconostoc mesenteroides* P-60 (8042), and the lactobacilli *pentosus* (124-2), *brassicae* (8041), *arabinosus* 17-5 (8014), *delbrückii* LD5 (9595), and *casei* ε (7469). Acid production was moderately good with all other

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[2] Selected because the rates of acid production were higher than those observed at 28° and 40° for some of the lactic acid bacteria.

strains except *Lactobacillus buchneri*. The response of fourteen of the organisms was further tested by measuring acid production in media containing 0.1 and 0.25 times the concentration of total amino acids and vitamins given in Table I. The results of these experiments are given in Table III. The rate and amount of acid production observed in the origi-

TABLE I

*Composition of Basal Medium**

Constituent	mg. per l.	Constituent	mg. per l.
dl-Alanine	667	Uracil	12.0
Asparagine, natural	667	Xanthine	12.0
l(+)-Arginine·HCl	667	Dextrose	20,000
l(−)-Cysteine·HCl	667	Sodium acetate	12,000
l(+)-Glutamic acid	667	Ammonium chloride	6,000
Glycine	667	KH_2PO_4	500
l(−)-Histidine·HCl·H_2O	667	K_2HPO_4	500
l(−)-Hydroxyproline	667	$MgSO_4·7H_2O$	200
dl-Isoleucine	667	$FeSO_4·7H_2O$	10
l(−)-Leucine	667	$MnSO_4·4H_2O$	10
dl-Lysine·HCl	667	NaCl	350
dl-Methionine	667	Thiamine hydrochloride	1.0
dl-Norleucine	667	Pyridoxine	1.6
dl-Norvaline	667	Pyridoxamine·2HCl	0.10
dl-Phenylalanine	667	Pyridoxal·HCl	0.10
l(−)-Proline	667	dl-Calcium pantothenate	2.0
dl-Serine	667	Riboflavin	2.0
dl-Threonine	667	Nicotinic acid	2.0
dl-Tryptophane	667	Biotin	0.005
l(−)-Tyrosine	667	p-Aminobenzoic acid	0.10
dl-Valine	667	Folic acid†	0.005
Adenine sulfate·2H₂O	13.8	Choline chloride	10.0
Guanine·HCl·2H₂O	13.0	Inositol	25.0

* All of the amino acids except proline and hydroxyproline were of c.p. or higher quality. Freedom of the constituents of the basal medium from contamination by amino acids other than norleucine and norvaline was shown conclusively by microbiological methods. The final solution was adjusted to pH 6.8.

† Product described previously (15).

nal medium were significantly lower for all of the organisms in the medium containing one-tenth the total amino acids and vitamins. In the medium containing one-fourth the total vitamins and amino acids the rate of acid production was lower for all strains except *Lactobacillus lycopersici* (4005), *Lactobacillus fermenti* 36 (9338), *Lactobacillus mannitopoeus*, and *Lactobacillus gayonii* (8289). In all but a few cases, maximum acid production

was essentially the same in the two media. Although it seemed probable that the nutritional quality of the original medium could be improved,

TABLE II

Rates of Acid Production

Organism*	Ml. of acid†				
L. mesenteroides (8293)‡ . . .	2.5 (10)	8.2 (25)	9.0 (31)	9.6 (52)	9.9 (99)
L. citrovorum (7013)‡ ..	2 5 (10)	8.2 (24)	9.7 (52)	9.9 (74)	9.1 (98)
L. citrovorum (797)‡ ...	2 1 (10)	7.9 (30)	9.1 (52)	9.5 (74)	9.0 (98)
L. mesenteroides (9135)‡ .	1.1 (9)	8 4 (29)	9.2 (50)	9.6 (73)	9.1 (98)
L. dextranicum (8086)‡	2.0 (10)	8 2 (30)	9.5 (52)	9.4 (74)	9.6 (98)
L. pentosus 124-2§	3 6 (11)	9.7 (22)	12.2 (30)	17.1 (78)	17.2 (97)
L. brassicae (8041)‡,‖	2.9 (8)	9 9 (21)	12.2 (29)	15 5 (53)	17.2 (77)
L. buchneri¶,‖	0 0 (24)	0.5 (48)	1.5 (91)	3 1 (142)	
L. arabinosus 17-5 (8014)‡	1.9 (6)	7.9 (12)	14.5 (22)	16.9 (55)	17.0 (79)
L. citrovorum (8082)‡	0 6 (23)	2.8 (29)	3.8 (50)	4.4 (73)	4.5 (98)
L. lycopersici (4005)‡,‖	0 0 (25)	2.9 (49)	8.7 (73)	9.1 (92)	9.0 (143)
L. dextranicum (8358)‡	1.1 (9)	4.5 (23)	5.8 (50)	6.1 (73)	5.5 (98)
L. dextranicum (8359)‡	1.4 (9)	7.9 (29)	9.1 (50)	9.3 (73)	8.8 (98)
L. fermenti 36 (9338)¶,‖	0.7 (7)	3.1 (12)	8.9 (23)	8.5 (56)	8.6 (80)
L. mannitopoeus¶, ‖	0.0 (10)	6.0 (20)	8.6 (28)	8.7 (52)	8.6 (76)
L. delbruckii LD5 (9595)‡, **	0.4 (16)	1.9 (23)	10.5 (41)	16.0 (64)	16.1 (134)
L. casei (7469)‡	0.8 (16)	3 3 (23)	11.8 (41)	16.2 (64)	15.6 (134)
L. gayoni (8289)‡, ‖	0.4 (10)	3 5 (25)	4.8 (31)	8 2 (52)	8.1 (75)
S. faecalis R (8043)§	2 5 (18)	4.5 (26)	6.4 (50)	8 0 (74)	8.4 (93)
L. citrovorum (8081)‡.	1.8 (9)	8.4 (23)	11.8 (50)	13.2 (73)	13.1 (98)
L. pentoaceticus (367)‡,‖	0 0 (10)	2 2 (19)	4.2 (28)	6.9 (52)	8.7 (76)
L. mesenteroides P-60 (8042)§ ..	1.0 (10)	7.0 (24)	12.1 (52)	14.9 (74)	14.6 (98)
L. brevis (8257)‡, ‖	0.0 (9)	1.3 (19)	2.6 (27)	5 4 (51)	7.4 (75)

* The numbers in parentheses are those given by the American Type Culture Collection.

† Given as ml. of 0 01 N NaOH to titrate 1 ml. of final solution. The values have been corrected for the blank titration. The numbers in parentheses refer to the incubation time in hours.

‡ Obtained from the American Type Culture Collection.

§ Obtained through the courtesy of Dr. E. E. Snell.

‖ Subcultured twice monthly on Bacto-tomato juice agar (Difco). Other organisms subcultured twice monthly on Bacto-yeast-dextrose agar (Difco).

¶ Obtained through the courtesy of Dr. V. H. Cheldelin

** Obtained through the courtesy of Dr. J. L. Stokes. Both cultures behaved identically in a large number of experiments.

especially for some of the organisms, it was considered to be sufficiently high to make worth while the determination of essential amino acids.

Maximum acid production by each of the twenty-three organisms was measured in the basal medium from which each of the twenty-one amino

TABLE III

Rates of Acid Production on Media Containing Three Concentrations of Total Amino Acids and Vitamins

Organism	Medium*	Ml of acid†				
L. pentosus 124-2	0.1	2.7 (6)	6.8 (22)	9.4 (30)	10.4 (50)	11.1 (71)
	0.25	3.4	11.2	13.3	15.8	17.0
	1.0	4.4	14.8	16.0	18.0	18.1
L. brassicae (8041)	0.1	1.9 (6)	5.0 (22)	5.9 (30)	7.2 (51)	8.1 (71)
	0.25	2 1	9.3	11.0	13.4	14.8
	1.0	4.2	14 2	15.8	18.0	18.6
L. buchneri	0.1	1.2 (7)	2 0 (27)	4.0 (96)	4 5 (120)	5.0 (146)
	0 25	1.3	2 1	4.1	4.9	5.5
	1 0	1.9	2 6	4 4	5.3	6 0
L. arabinosus 17-5 (8014)	0 1	3 0 (6)	8.8 (22)	10.0 (30)	14 5 (71)	16.0 (118)
	0.25	4 0	10 6	12.5	17 2	18.0
	1.0	5.6	17.5	18.7	19.2	18.4
L. lycopersici (4005)	0.1	4 1 (21)	5.5 (29)	6.8 (50)	7.6 (70)	8.4 (118)
	0 25	4.7	7.1	10.1	11.0	11.0
	1.0	5.5	8.5	10.7	10.8	10.5
L. fermenti 36 (9338)	0.1	2.1 (6)	7.2 (22)	7.7 (30)	8.5 (51)	8.7 (71)
	0.25	2 3	10.9	10.5	10.6	10 5
	1 0	2.9	10 9	10.5	10.5	11.0
L. mannitopoeus	0.1	8.0 (21)	8 9 (29)	10.2 (50)	10.4 (70)	10.0 (118)
	0 25	10.4	10.2	10 3	10.3	9.9
	1 0	10 7	10.6	10 4	10 7	10.2
L. delbruckii LD5 (9595)	0 1	3 2 (16)	4.7 (24)	9 2 (44)	11.8 (65)	13.8 (112)
	0 25	3 7	6 2	11 7	14 5	18.2
	1 0	5 9	10.3	16.2	18 5	18.7
L. casei (7469)	0 1	2 7 (16)	4 2 (24)	9 1 (44)	11 6 (65)	14 1 (112)
	0 25	3.1	5.4	10 6	13 5	18.3
	1.0	5.0	8 3	15.2	18 5	18.6
L. gayoni (8289)	0.1	5.6 (21)	6.2 (29)	7.6 (50)	8.0 (70)	7.9 (118)
	0 25	7.6	9.1	10.5	10.6	10.2
	1.0	7.6	9.7	10.9	11.1	10.5
S. faecalis R (8043)	0.1	1.8 (18)	2.6 (26)	2.8 (44)	3.6 (65)	4.5 (112)
	0 25	3.8	4.7	6.3	6.8	7.0
	1.0	5.1	6.4	8 5	9.4	10.2
L. pentoaceticus (367)	0.1	2.1 (16)	2 3 (24)	3.3 (44)	3.8 (65)	5.2 (112)
	0.25	2 9	3 9	5.1	6.5	7.5
	1.0	5.0	6 6	9.8	10.4	10.3
L. mesenteroides P-60 (8042)	0.1	4.1 (21)	4.6 (29)	7.1 (50)	8.2 (70)	9.1 (118)
	0.25	8.2	9.4	12.7	13.7	14.3
	1.0	10.9	13.2	15.4	16.8	16.8
L. brevis (8257)	0.1	1.4 (7)	2.4 (27)	3.0 (48)	3.4 (96)	3.4 (96)
	0.25	1.6	3.4	4.7	5.7	6.1
	1.0	1.6	6.3	8.9	10.5	10.2

<div align="center">TABLE III—Concluded</div>

* The media contained 0.1, 0.25, and 1.0 times the concentration of total amino acids and vitamins given in Table I. Crystalline folic acid obtained from the Lederle Laboratories through the courtesy of Dr. S. M. Hardy was employed in these media in place of the folic acid concentrate referred to in Table I.

† Given as ml. of 0.01 N NaOH to titrate 1 ml. of final solution. The numbers in parentheses refer to the incubation time in hours.

<div align="center">TABLE IV</div>

<div align="center">Maximum Acid Production in Media Lacking Amino Acid*</div>

Amino acid absent from medium	L. mesenteroides (8293)		L. citrovorum (7013)		L. citrovorum (797)		L. mesenteroides (9135)		L. dextranicum (8086)	
	Volume NaOH	Time	Volume NaOH	Time	Volume NaOH	Time	Volume NaOH	Time	Volume NaOH	Time
	ml.	hrs.	ml.	hrs.	ml.	hrs.	ml.	hrs.	ml.	hrs.
Glutamic acid	**1.9**	75	**1.6**	74	**1.6**	98	**2.2**	50	**1.1**	74
Valine	**1.2**	99	**1.2**	74	**0.8**	74	**0.5**	73	**0.5**	52
Isoleucine	9.4	99	6.7	98	6.4	98	**1.9**	73	**0.9**	98
Leucine	9 3	114	5.3	92	9.4	114	7.8	114	9.2	114
Methionine	5.8	99	6.3	98	4.8	74	4.4	73	**3.4**	98
Arginine	9.7	52	9.7	52	9.8	74	9.7	73	9.8	52
Tryptophane	9.8	52	9.7	74	9.5	74	9.5	73	9.5	98
Cysteine	9.6	75	9.3	52	9.8	52	6.6	73	9.4	52
Histidine	8.5	52	8.5	74	8.1	74	9.5	73	8.3	98
Threonine	10.1	99	9.4	74	9.1	74	9.5	73	9.8	98
Phenylalanine	9.5	52	10.1	74	9.6	74	9.3	73	9.4	52
Tyrosine	9.8	52	9.7	74	9.7	74	9.5	73	9.6	98
Glycine	9.9	52	10.0	74	9.7	74	9.6	73	9.9	52
Asparagine	9.8	52	9.8	52	9.3	52	5.2	73	9.6	98
Lysine	9 9	52	9.8	52	9.9	74	9.6	73	9.9	52
Serine	9.8	75	9.4	74	9.3	74	9.6	73	9.6	52
Alanine	9.8	52	9.9	52	9.4	74	3.9	50	9.5	52
Proline	9.6	52	9.2	74	9.5	52	9.6	73	9.3	74
Norleucine	9.8	52	10.0	74	9.9	74	9.6	73	9.6	52
Hydroxyproline	9 8	52	10.2	74	9.9	74	9.8	73	10.1	74
Norvaline	9.6	53	9.8	74	9.8	74	9.5	73	9.5	52
Control	9.9	99	9.9	74	9.5	74	9.6	73	9.5	52

* The acid production is given as ml. of 0.01 N NaOH to titrate 1 ml. of the final solution. The values have been corrected for the blank titration. The values shown in bold-faced type of less than one-fourth the maximum control titration are considered to be indicative of essential amino acids. Since the inocula were unusually large, relatively high blank titrations were not unexpected. According to the observation of Virtanen and Karström (16), glucose is fermented by cells *per se* even in the non-living, dried state.

acids had been omitted in turn. The results of these experiments are shown in Table IV. The microorganisms are arranged in order of their

fastidiousness for the amino acids, and the latter are listed approximately in order of their indispensability for the strains tested. For *Leuconostoc mesenteroides* (8293), the least fastidious strain, only glutamic acid and valine were essential, while *Lactobacillus brevis* (8257), the most fastidious, required fifteen amino acids. Glutamic acid and valine were required by all of the organisms, while isoleucine was essential for all but three and

TABLE IV—*Continued*

Amino acid absent from medium	L. brassicae (8041)		L. buchneri		L. pentosus 124-2		L. arabinosus 17-5 (8014)		L. citrovorum (8082)		L. lycopersici (4005)	
	Volume NaOH	Time	Volume NaOH	Time	Volume NaOH	Time	Volume NaOH	Time	Volume NaOH	Time	Volume NaOH	Time
	ml.	*hrs.*	*ml.*	*hrs.*	*ml.*	*hrs.*	*ml.*	*hrs.*	*ml.*	*hrs.*	*ml.*	*hrs.*
Glutamic acid	0.6	100	0.1	142	1.6	100	2.4	150	0.5	98	0.0	143
Valine	0.4	80	0.0	142	0.4	80	1.7	55	0.5	98	0.0	143
Isoleucine	0.4	100	0.0	142	0.6	100	2.0	150	0.4	98	0.0	143
Leucine	0.5	100	0.0	142	0.9	100	2.2	150	0.1	66	0.0	143
Methionine	7.2	80	0.6	142	14.8	80	4.4	55	1.3	73	4.5	143
Arginine	17.0	80	3.0	142	17.1	80	12.6	55	0.7	98	10.0	143
Tryptophane	16.8	80	2.9	142	16.6	80	2.7	80	1.0	98	9.1	92
Cysteine	1.1	100	0.0	142	2.1	100	15.1	55	4.5	98	0.1	143
Histidine	17.0	80	3.3	142	17.3	80	17.1	55	0.6	98	9.6	92
Threonine	17.0	80	2.9	142	16 4	80	16.6	55	3.1	98	9.5	92
Phenylalanine	17.2	80	3.0	142	17.1	80	11.4	55	5.4	98	9.4	92
Tyrosine	17.5	80	3.3	142	16.8	80	14 0	55	4.3	98	9.5	92
Glycine	17.0	80	3 0	142	17.3	80	17.1	55	3.5	98	9.6	92
Asparagine	17.3	80	0.1	142	17.3	80	17.1	55	3.8	98	0.1	143
Lysine	17.3	80	3.4	142	17.1	80	17.1	55	3.1	98	9.4	93
Serine	17.3	141	2.8	142	17.1	140	17.0	140	3.6	98	9.4	112
Alanine	17.0	80	2.7	142	17.0	80	16.8	55	3.7	98	0.1	143
Proline	16.8	141	2.7	142	17.1	140	16.7	140	3.6	98	9.6	64
Norleucine	17.3	80	3.2	142	17.2	80	17.0	55	4.3	98	9.1	92
Hydroxyproline	17.0	80	3.1	142	17.4	80	17.0	55	4.2	98	9.4	92
Norvaline	17.4	80	3.0	142	17.2	80	17.1	55	4.1	98	9.4	92
Control	17.2	80	3.1	142	17.2	100	17.0	55	4.5	98	9.1	92

leucine for all but four strains. The number of strains which required each of the following amino acids are shown in parentheses: methionine (10), arginine (10), tryptophane (13), cysteine (12), histidine (9), threonine (6), phenylalanine (7), tyrosine (8), glycine (4), aspartic acid (4), lysine (4), serine (2), and alanine (3). Norleucine, norvaline, proline, and hydroxyproline were without pronounced effect on acid production by any of the microorganisms. If any other constituents of the basal medium

were contaminated with one or more of these four amino acids, the non-essentiality observed for the latter would be invalidated. The quality of the amino acids employed in the present experiments is indicated in the foot-note to Table I.

That norleucine and norvaline were found to be non-essential was not unexpected, since these amino acids may not exist in protein materials. Because the essentiality of proline and hydroxyproline might not have been

TABLE IV —*Continued*

Amino acid absent from medium	L dex-transcum (8358)		L dex-transcum (8359)		L fer-menti 36 (9338)		L manni-topoeus		L delbrückii LD5 (9595)		L casei (7469)	
	Volume NaOH	Time	Volume NaOH	Time	Volume NaOH	Time	Volume NaOH	Time	Volume NaOH	Time	Volume NaOH	Time
	ml.	*hrs*	*ml*	*hrs*	*ml*	*hrs*	*ml*	*hrs*	*ml*	*hrs*	*ml*	*hrs.*
Glutamic acid	0.9	97	0.7	50	0.8	56	0.0	146	0.0	144	0.0	150
Valine	0.6	73	0.5	50	0.5	80	0.1	146	0.1	144	0.0	150
Isoleucine	1.5	73	0.5	50	0.6	56	0.0	146	0.4	144	0.0	150
Leucine	1.0	66	0.4	114	0.6	56	0.2	146	0.1	144	0.0	150
Methionine	2 2	73	2.2	50	0.4	80	0.1	52	11 8	93	16.8	150
Arginine	2 9	50	9 6	72	9.1	23	0.2	146	1.1	144	0.9	150
Tryptophane	4.5	73	0.8	72	0.6	56	0.2	146	1.6	144	1.2	150
Cysteine	1.2	97	0.8	72	9.1	56	9 1	76	2.6	144	2.2	150
Histidine	1.3	50	0.7	50	6.0	80	9 0	76	11.2	93	15 5	150
Threonine	1.1	50	7.1	72	9 1	80	9 1	76	16 2	93	17 9	150
Phenylalanine	3 5	73	4 4	72	0.5	56	0.1	146	3 5	93	5 5	150
Tyrosine	4 8	73	7 0	72	1.1	80	0.5	52	4 2	144	3.9	150
Glycine	6.0	73	9 7	72	9 5	23	9.0	52	16 7	93	18 0	150
Asparagine	6.2	73	9 2	72	9.1	23	9.0	52	17 5	93	17 7	150
Lysine	5.8	73	9 2	72	9 0	23	9 0	52	13 5	93	17 4	150
Serine	3 8	.50	8.0	72	9.0	116	9 8	115	0.5	137	1.8	141
Alanine	6 8	73	9 0	50	8.7	56	9 0	76	13.2	93	16 8	150
Proline	4 4	73	9 2	72	9 1	116	9 8	115	15 5	137	16 8	141
Norleucine	6 1	73	9 4	72	9 1	23	8 8	52	12.7	93	17.1	150
Hydroxyproline	6.1	73	9.3	72	9.0	23	8 9	52	12.5	93	17 3	150
Norvaline	5.9	73	9.1	50	9.4	23	9 0	50	12.2	93	17.4	150
Control	6.1	73	9.3	72	9 0	23	8.7	52	12.7	93	17.2	150

exhibited in the experiments described, further studies were made of these amino acids. This problem was of particular interest because it had been found earlier (3) that proline was essential for *Leuconostoc mesenteroides* P-60 grown in a basal medium of different composition than that employed in the present experiments. The requirements of all of the organisms were reinvestigated, therefore, with the basal medium given in Table I, but modified to contain no proline and hydroxyproline or markedly lower

concentrations of these amino acids. The rates of acid production by six of the microorganisms on four types of modified media are shown in Table V. The experimental data for seventeen of the strains were not included in Table V, since the rates of acid production were not altered significantly even with both proline and hydroxyproline absent from the basal medium. Significant amounts of acid were formed by *Lactobacillus lycopersici* (4005), *Leuconostoc dextranicum* (8359), *Lactobacillus pento-*

TABLE IV—*Concluded*

Amino acid absent from medium	L gayonii (8289)		S faecalis R (8043)		L citrovorum (8081)		L. pento-aceticus (367)		L mesen-teroides P-60 (8042)		L. brevis (8257)	
	Volume NaOH	Time	Volume NaOH	Time	Volume NaOH	Time	Volume NaOH	Time	Volume NaOH	Time	Volume NaOH	Time
	ml	hrs	ml	hrs	ml.	hrs	ml	hrs	ml	hrs	ml.	hrs.
Glutamic acid	0.4	99	0.2	168	1.0	98	0.5	94	0.4	74	0.0	169
Valine	0.4	99	0.4	168	0.9	73	0.1	170	0.5	74	0.0	169
Isoleucine	0.3	52	0.6	168	1.0	98	0.0	170	0.4	74	0.0	169
Leucine . .	0.1	66	0.4	168	1.2	92	0.1	170	0.4	92	0.0	169
Methionine .	0.5	52	0.9	168	3.9	73	1.2	170	3.5	74	0.2	169
Arginine	0.3	52	0.9	168	1.1	98	0.5	170	0.5	51	0.2	169
Tryptophane	0.3	52	1.2	93	2.6	98	0.7	170	1.2	98	0.0	169
Cysteine .	9 0	75	6 0	93	2.0	73	0.4	94	1.7	98	0.0	169
Histidine	2.0	99	0.9	168	1.8	98	0.7	170	1.0	98	0.2	169
Threonine	3 9	99	0.4	168	0.7	98	0.0	170	0.2	98	0.0	169
Phenylalanine	0.4	52	5 6	93	2.0	98	1.2	76	3.7	98	1.0	169
Tyrosine	0.4	52	1.9	168	3 7	98	2.0	170	2.8	98	1.2	169
Glycine	9 2	75	2.9	168	1.8	98	2.0	170	2.2	98	1.0	169
Asparagine .	9.0	75	8 1	93	4.7	98	5.6	76	0.8	74	0.1	169
Lysine	8.6	52	0.5	168	11 7	73	0.9	170	0.6	98	0.0	169
Serine . .	9.1	52	3.5	137	4.5	98	8 4	139	10.7	98	4.9	140
Alanine	8.4	75	1.4	168	1.2	98	5.6	76	7.9	98	4.2	75
Proline	9.0	52	8,1	137	12.5	98	6.1	114	13.5	98	4.7	140
Norleucine . .	8.5	52	8 7	93	14.1	73	8.5	76	14.7	98	6.4	75
Hydroxyproline .	9 3	75	8.1	93	13 3	98	8.7	76	14.9	98	7.1	75
Norvaline	9.3	52	8.1	93	14 3	98	9.0	76	14.2	98	6.4	75
Control...	8.2	52	8 4	93	13.2	73	8.7	76	14.9	74	7.4	75

aceticus (367), and *Leuconostoc mesenteroides* P-60 (8042), even in the absence of both proline and hydroxyproline, although acid was produced more slowly by the last two than the first two strains. . Acid production was delayed by the last two organisms when proline alone was omitted from the medium. The slow rate of acid production by *Leuconostoc citro-vorum* (8081), and especially by *Lactobacillus brevis* (8257), on a medium with only proline absent may indicate either a slow synthesis of proline or the presence of small amounts of proline in the sample of hydroxyproline.

TABLE V

Rates of Acid Production on Media Lacking or Deficient in Proline and Hydroxyproline

Organism	Medium No.*	Ml. of acid†					
L. lycopersici (4005)	1	1.6 (15)	2.5 (24)	7.3 (40)	11.3 (64)	11.2 (112)	10.9 (137)
	2	1.6	2.3	4.5	11.2	11.2	10.9
	3	1.5	2.3	6.8	11.0	10.9	10.8
	4	1.7	2.4	5.8	11.0	11.3	10.9
L. dextranicum (8359)	1	6.7 (18)	8.5 (37)	9.4 (64)	9.1 (86)	9.2 (111)	9.1 (131)
	2	6.8	8.6	9.3	9.2	9.2	8.9
	3	6.3	8.1	8.8	8.3	8.4	8.2
	4	6.3	7.9	8.6	8.3	8.2	8.1
L. citrovorum (8081)	1	10.7 (20)	13.5 (47)	14.1 (69)	14.0 (94)	13.8 (105)	
	2	10.9	13.4	14.0	14.0	13.7	
	3	3.2	4.9	6.3	7.4	7.7	
	4	2.4	2.7	2.8	3.0	3.2	
L. pentoaceticus (367)	1	4.0 (17)	5.8 (26)	8.9 (42)	10.5 (66)	10.5 (114)	10.4 (139)
	2	4.2	5.7	8.8	10.5	10.6	10.4
	3	1.8	2.0	4.9	7.6	8.2	6.0
	4	1.7	1.8	2.1	6.9	11.4	3.1
L. mesenteroides P-60 (8042)	1	6.7 (18)	9.0 (27)	12.5 (43)	15.1 (67)	17.0 (115)	16.8 (140)
	2	6.4	8.6	12.1	14.4	16.5	16.6
	3	1.8	1.9	2.7	6.8	9.0	8.7
	4	1.8	1.9	3.4	6.3	11.2	6.8
L. brevis (8257)	1	3.1 (18)	4.4 (27)	6.7 (43)	9.4 (67)	11.3 (115)	10.9 (140)
	2	3.3	4.7	6.9	9.6	10.7	11.1
	3	1.9	1.9	2.2	2.7	3.6	3.7
	4	1.8	1.8	2.0	2.1	2.2	2.3

* The media were those given in Table I, except that Medium 1 contained one-twentieth the concentrations of proline and hydroxyproline, Medium 2 contained no hydroxyproline and one-tenth the concentration of proline, Medium 3 contained no proline and one-tenth the concentration of hydroxyproline, and Medium 4 contained no proline or hydroxyproline.

† Given as ml. of 0.01 N NaOH to titrate 1 ml. of final solution. The figures in parentheses refer to the incubation times in hours.

The eleven amino acids found to be essential for *Lactobacillus fermenti* 36 (9338) on the medium given in Table I are the same as those reported earlier (14) to be required on a medium of different composition. It was shown previously (3) that seventeen amino acids were required by *Leuconostoc mesenteroides* P-60 (8042), but it has been found subsequently (unpublished data) that alanine, proline, and serine are synthesized after prolonged incubation on this medium. Similarly, alanine, glycine, proline, and serine are synthesized rapidly, while phenylalanine and tyrosine are synthesized slowly, on the present enriched medium. Isoleucine was shown to be essential for *Lactobacillus casei* ε (7469) on the present medium as well as that employed by Baumgarten *et al.* (17). Since McMahan and Snell (18) found that isoleucine was not essential for the growth of *Lactobacillus casei*, it would appear that this amino acid was synthesized on the basal medium employed or, more probably, that the medium was contaminated with isoleucine. It is of interest that Hegsted and Wardwell (19) have reported that some commercial samples of *dl*-leucine contained appreciable amounts of isoleucine.

Twelve amino acids (glutamic acid, valine, isoleucine, leucine, methionine, arginine, tryptophane, histidine, threonine, tyrosine, lysine, and alanine) were found to be essential for *Streptococcus faecalis* R (8043) in the present experiments. With the exception of alanine and tyrosine, all of these amino acids are required by this organism, according to Snell and Guirard (20), Stokes *et al.* (5), and Greenhut *et al.* (6), although only seven of the amino acids were stated to be essential by the first authors, nine by the second, and ten by the third. Other amino acids which these workers considered to be essential were aspartic acid and serine (20), phenylalanine (5), and aspartic acid, serine, and cystine (6). It is of further interest that *Streptococcus faecalis* R was used for the determination of histidine by Baumgarten *et al.* (17), threonine by Greenhut *et al.* (6), and, except phenylalanine, all of the ten essential amino acids by Stokes *et al.* (5). Although it would appear from the foregoing discussion that *Streptococcus faecalis* R has amino acid requirements which vary with the composition of the basal medium, it may be that some of the stated conclusions are vitiated because of unsuspected amino acid impurities.

The amino acid requirements of *Lactobacillus mannitopoeus*, *Lactobacillus lycopersici* (4005), and *Lactobacillus buchneri*, determined by the present workers, were considerably different from those reported ·by Wood *et al.* (8). It is difficult to ascertain the extent to which these differences may be due to the widely different composition of the basal media, amino acid impurities in the nutrient substances, and differences in the strains of the organisms employed in the two laboratories.

The amino acids given in bold-faced type in Table IV were essential up to

and beyond the incubation period required for maximum acid production in control experiments. Amino acids in this category are of particular interest because of the probability that they may be determined more accurately in biological materials than amino acids which are required by organisms only during more restricted incubation periods. It has been found worth while, however, to provide procedures for certain amino acids such as threonine (21), which could be determined with satisfactory accuracy only within the stated time of incubation. For this reason, the amino acid requirements of the organisms employed in the present experiments have been further investigated. Data representing the maximum *drop* in acid production resulting from the omission of each amino acid in turn from the complete basal medium (Table I) are shown in Table VI. The gradations in partial amino acid requirements, which are indicated by these data, were not revealed by the studies (Table IV) of maximum acid production. The times of incubation over which the amino acids, corresponding to the values (given in bold-faced type) for the maximum drop in acid production, might be determined microbiologically are given in Table VI. In a limited number of cases, the omission of certain amino acids from the basal medium resulted in an increased rate of acid production without affecting the amount of acid finally produced. From the experimental results shown in Table VII, it may be concluded that these amino acids inhibited acid formation by the stated organisms. Although amino acid inhibitions occur infrequently in media of relatively high nutritional quality, they have been observed previously by Hutchings and Peterson (13), Dunn *et al.* (3, 14), Shankman (10), McMahan and Snell (18), and Snell and Guirard (20). In another type of inhibition, reported by Fox and coworkers (22, 23), excessive amounts of unnatural antipodes of certain amino acids inhibited the growth-promoting effects of the natural forms.

It may be noted from the experimental results given in Tables II to IV and VI that there is little correlation between the nutritional properties of different organisms of the same species. For example, fifteen amino acids were essential for *Leuconostoc mesenteroides* P-60 (8042), but only two amino acids were required by *Leuconostoc mesenteroides* (8293). Of all the cultures studied, the only members of the same species which behaved similarly were *Leuconostoc citrovorum* (7013) and *Leuconostoc citrovorum* (797). On the other hand, the visible growth of *Lactobacillus pentosus* 124-2 and *Lactobacillus brassicae* (8041), as well as the rate and amount of acid production by these strains, was almost identical on all of the basal media. Although acid production by *Lactobacillus brassicae* (8041), but not by *Lactobacillus pentosus* 124-2, was somewhat retarded when pyridoxine, pyridoxal, and pyridoxamine were omitted from the medium,

both organisms responded alike to the other vitamins (unpublished data). For these reasons, it was tentatively concluded that these strains probably were of the same species.

Even more marked was the apparent similarity in the nutritional requirements of *Lactobacillus delbrückii* LD5 (9595) and *Lactobacillus casei*

TABLE VI

Maximum Drop in Acid Production Resulting from Omission of Amino Acid*

Amino acid absent from medium	L. mesenteroides (8293)		L. citrovorum (7013)		L. citrovorum (797)		L. mesenteroides (9135)		L. dextranicum (8086)	
	Volume NaOH	Time	Volume NaOH	Time	Volume NaOH	Time	Volume NaOH	Time	Volume NaOH	Time
	ml	hrs	ml	hrs.	ml.	hrs.	ml	hrs.	ml	hrs.
Glutamic acid	*7 9*	99	*8.2*	74	*7.9*	74	*8.1*	73	*8.4*	52
Valine	*8 5*	99	*8.6*	52	*8.5*	74	*8.8*	73	*8.8*	52
Isoleucine	4 3	25	**6.6**	24	**6.3**	24	*7.6*	50	8.7	52
Leucine	1.4	25	0 7	42	1 1	25	2.6	66	0.4	25
Methionine	5 4	31	5 3	30	4.9	24	5.6	29	**6.2**	52
Arginine	0.5	25	0 6	10	0.3	10	3.1	23	0.4	24
Tryptophane	0 4	25	0 3	10	0.1	24	0 9	23	0.5	10
Cysteine	0.6	31	0.6	10	0.8	10	2 9	50	0.6	10
Histidine	1.8	25	1 8	24	1.7	24	1.1	23	1.9	30
Threonine	3.7	25	3 4	30	2.9	24	0.8	23	1.7	30
Phenylalanine	0 8	25	5.1	24	0.9	24	0.3	9	0.5	10
Tyrosine	0.8	10	0.6	10	0.1	24	0.4	23	0 5	10
Glycine	0.3	25	0 4	10	0.2	24	0.5	23	0.1	10
Asparagine	0 7	25	0 4	24	0.1	10	**7.3**	29	0.1	10
Lysine	0 1	25	0 2	10	0.0		0.2	23	0.6	24
Serine	1 2	25	0 9	10	0.7	24	1.1	23	0.7	24
Alanine	0.1	31	0 4	10	0.0		5 3	73	0.4	10
Proline	0 7	25	0.9	24	0.5	24	0.6	23	0.3	24
Norleucine	0.1	10	0 1	10	0.0		0 0		0.0	
Hydroxyproline	0.1	25	0.3	24	0.0		0.2	23	0.0	
Norvaline	0.1	25	0.1	10	0.0		0.0		0.2	10

* Each volume of NaOH represents the maximum difference between the volume of 0 01 N NaOH required to titrate 1 ml. of final solution containing the complete medium and that with one amino acid absent. The values in italics (corresponding to those given in bold-faced type in Table IV) or in bold-faced type are at least two-thirds the maximum difference.

ε (7469). Not only were the rates and amounts of acid production, the visible growth, and the vitamin requirements (unpublished data) nearly identical on all of the media, but also the nearly quantitative similarity observed in the inhibitions (Table VII) by four amino acids was found only in these two of the twenty-three organisms studied. It seemed desirable

to investigate these strains further in order to supplement the nutritional studies of other workers.[3] It was found (unpublished data) that both strains[4] responded identically in producing acid from lactose in 24 hours, in producing acid from mannitol in 48 hours, and in coagulating milk in 72 hours. According to Bergey's (26) classification, these tests should be positive for *Lactobacillus casei* but negative for *Lactobacillus delbrückii*. In view of these findings it appears probable that the cultures of these

TABLE VI—*Continued*

Amino acid absent from medium	L. pentosus 124-2		L. brassicae (8041)		L. buchneri		L. arabinosus 17-5 (8014)		L. citrovorum (8082)		L lycopersici (4005)	
	Volume NaOH	Time	Volume NaOH	Time	Volume NaOH	Time	Volume NaOH	Time	Volume NaOH	Time	Volume NaOH	Time
	ml.	hrs.	ml.	hrs.	ml	hrs.	ml.	hrs.	ml	hrs.	ml	hrs.
Glutamic acid	15.7	80	16.6	80	2.9	142	15.5	60	4.0	73	9.2	92
Valine	16.8	100	16.8	80	3.4	142	16.2	60	3.9	73	9.2	92
Isoleucine	16.7	80	16.9	80	3.5	142	15.8	60	3.8	73	9 6	49
Leucine	16.4	80	16.8	80	3 4	142	15.7	60	2.6	114	9.4	49
Methionine	6.9	22	9.7	30	2.4	142	13.0	40	3.0	.98	7.1	73
Arginine	2.9	22	5.5	20	0.4	142	10.8	20	3.6	73	3 9	73
Tryptophane	2.0	11	1.0	8	0.5	91	15.0	60	3.3	73	0 5	73
Cysteine	15.1	100	16.1	80	3.4	142	9.4	20	0.2	9	9 1	92
Histidine	0.9	11	0.6	8	0.2	91	1.6	14	3.7	73	1.7	49
Threonine	0.5	11	5.9	20	0.2	142	4.1	16	1.8	50	2.9	73
Phenylalanine	1.7	11	1.4	8	0.2	142	7.9	20	0.2	23	0 1	25
Tyrosine	0.6	11	0.2	8	0.2	91	5.9	20	0.4	29	0.0	
Glycine	1.6	11	5.5	20	0.4	91	4.5	12	1.4	73	0.5	49
Asparagine	0.2	11	1.7	8	3.2	142	2.9	12	1.2	29	9.2	92
Lysine....	0.6	11	0.6	8	0.4	24	2.4	12	1.3	29	0.7	73
Serine	1.4	26	3.4	20	0.5	142	0.8	20	1.0	29	0.6	24
Alanine	0.1	22	0.1	8	1.1	142	0.5	12	0.6	50	9.2	92
Proline	0.0		0.6	20	0.4	142	1.0	20	1.8	29	0.2	24
Norleucine	0.0		0.0		0.5	91	0.0		0.2	23	0 1	25
Hydroxyproline	0 5	11	0.4	8	0.0		0.8	12	0.7	9	0.1	449
Norvaline	0.4	11	0.4	8	0.1	91	0.4	12	0.1	23	0.1	9

organisms represent closely similar, if not identical, varieties of the same species. It is of interest in this connection that the identity as *Lactobacillus casei* of organisms described in the literature as *Lactobacillus delbruckii*

[3] Comprehensive reviews have been given recently by Knight (24) and by Peterson and Peterson (25).

[4] Two cultures of *Lactobacillus delbrückii* LD5 (9595), one obtained from Dr. J. L. Stokes and the other from the American Type Culture Collection, responded identically in the nutrition experiments.

LD5 and *Lactobacillus bulgáricus* 05 has been established by taxonomic studies reported recently by Rogosa (27). It was found with authentic strains of these organisms that, unlike *Lactobacillus casei*, neither *Lactobacillus delbrückii* nor *Lactobacillus bulgaricus* grew in defined media containing all known B vitamins and amino acids.

Stokes and Gunness (28) encountered some difficulties in obtaining data as highly accurate as might be desired in determining aspartic acid

TABLE VI—*Continued*

Amino acid absent from medium	L. dextranicum (8358)		L. dextranicum (8359)		L. fermenti 36 (9338)		L. mannitopoeus		L. delbrückii LD5 (9595)		L. casei (7469)	
	Volume NaOH	Time	Volume NaOH	Time	Volume NaOH	Time	Volume NaOH	Time	Volume NaOH	Time	Volume NaOH	Time
	ml	hrs	ml	hrs	ml.	hrs.	ml	hrs.	ml.	hrs	ml.	hrs.
Glutamic acid	5.2	73	8.6	73	8.5	23	8.7	28	12.7	93	17.2	150
Valine	5.3	73	8.7	73	8.5	23	8.9	28	12.8	93	17.0	150
Isoleucine	4.3	73	8.5	73	8.5	23	9.0	28	12.6	93	17.1	150
Leucine	5.4	114	8.6	42	8.5	23	8.9	28	12.7	93	17.1	150
Methionine	3.7	73	6.9	73	8.7	23	8.7	28	1.1	74	0.5	50
Arginine	2.9	23	0.7	23	8.1	23	8.7	28	12.0	93	16.1	150
Tryptophane	1.3	73	8.2	73	8.6	23	8.7	28	11.5	93	15.7	150
Cysteine	4.9	73	8.4	73	2.9	23	8.1	28	11.1	93	14.7	150
Histidine	4.8	73	8.5	73	8.4	23	8.2	28	1.5	93	1.5	150
Threonine	4.7	73	3.0	23	8.5	23	8.9	28	0.0		0.1	18
Phenylalanine	2.4	73	4.9	50	8.6	23	8.9	28	9.2	93	11.5	150
Tyrosine	1.1	73	2.5	29	8.2	23	8.4	28	10.0	93	13.1	150
Glycine	0.0		0.4	23	0.1	12	1.9	28	0.0		0.5	30
Asparagine	0.0		0.6	23	0.0		2.2	20	0.4	25	2.4	50
Lysine	0.1	9	0.3	23	0.1	12	2.5	20	0.2	25	0.2	18
Serine	2.0	73	2.2	23	0.1	28	0.2	28	12.1	137	16.6	116
Alanine	0.0		0.2	23	3.0	23	8.9	28	0.0		0.1	18
Proline	0.8	23	0.4	23	1.2	19	0.0		0.2	24	0.4	28
Norleucine	0.0		0.3	23	0.0		1.0	20	0.4	25	0.6	50
Hydroxyproline	0.0		0.1	23	0.4	12	0.0		0.4	25	0.4	50
Novaline	0.0		0.1	9	0.2	7	0.1	20	0.6	73	0.4	50

and serine with *Lactobacillus delbrückii* LD5 (9595), with a basal medium essentially the same as that on which Hutchings and Peterson (13) found these amino acids to be essential for *Lactobacillus casei* ε. Although evidently these amino acids are required under some conditions for the growth of *Lactobacillus casei* ε (and hence the strain, referred to as *Lactobacillus delbrückii* LD5), the requirement at least for aspartic acid is nonspecific, since good growth on the present medium resulted in the absence of

aspartic acid and asparagine, and inhibitions occurred in the presence of asparagine. In searching for the essential substances which may have been lacking in the medium employed by Stokes and Gunness and by Hutchings and Peterson, it was ascertained that neither acid nor visible growth was produced up to 64 hours incubation when both uracil and asparagine were omitted from the enriched medium. Incubation up to 134 hours was required before either acid or growth was observed. The omission of uracil

TABLE VI—*Concluded*

Amino acid absent from medium	L gayonii (8289)		S faecalis R (8043)		L citrovorum (8081)		L pentoaceticus (367)		L mesenteroides P-60 (8042)		L brevis (8257)	
	Volume NaOH	Time	Volume NaOH	Time	Volume NaOH	Time	Volume NaOH	Time	Volume NaOH	Time	Volume NaOH	Time
	ml.	hrs	ml	hrs.	ml.	hrs.	ml.	hrs	ml	hrs	ml	hrs
Glutamic acid	7.8	52	7 7	74	12.3	73	8 5	76	14 4	74	7.5	75
Valine	7 9	52	7 7	74	12.1	73	8 9	76	14 2	74	7 6	75
Isoleucine	7 6	52	7 6	74	12.1	73	8 7	76	14 2	74	7.5	75
Leucine	8 2	66	7.7	74	12 0	73	8 7	76	14.0	74	7.4	75
Methionine	7 5	52	7.1	74	9 1	73	8 1	76	11.2	74	7.2	75
Arginine	7 6	52	7 6	74	12.0	73	8.5	76	14.1	74	7.4	75
Tryptophane	7.6	52	7 0	74	10 8	73	8 2	76	13 5	74	7.4	75
Cysteine	3.6	31	2 9	26	11.1	73	8.5	76	13 4	74	7 5	75
Histidine	7.8	52	7.1	74	11.4	73	8 3	76	13.8	74	7.2	75
Threonine	7.3	52	7.7	74	12.2	73	8.9	76	14.3	74	7.6	75
Phenylalanine	7 6	52	4 4	26	11.5	73	7 3	76	11 6	74	6.6	75
Tyrosine	7.6	52	6.6	74	9.5	73	7 7	76	12 3	74	6.5	75
Glycine	0.6	25	6.0	93	11.9	73	7.7	76	13 5	74	7.0	75
Asparagine	0 0		4.1	26	9.3	73	4.4	28	13 8	74	7 5	75
Lysine	0.5	31	7.6	74	1.4	9	8.7	76	14.0	74	7.4	75
Serine	0.5	25	5.2	40	9.2	73	6.1	42	6.4	52	6.6	67
Alanine	1.1	31	7.6	74	12.3	73	3.0	76	6.7	52	3.1	75
Proline	0.6	31	1.7	24	1.6	23	4.1	42	1.2	24	2.6	75
Norleucine	0.4	31	0 0		0.0		0.8	19	0.6	24	1 0	75
Hydroxyproline	0.6	31	1.4	18	0.1	23	0.5	28	1 0	24	0 7	51
Norvaline	0.4	25	0 9	26	0.1	23	0.6	28	1.0	24	1 0	75

alone had no effect on acid production. On the other hand, the omission of any other substance,[5] present in the enriched medium but lacking in that of the authors quoted, had no effect on the asparagine requirement. In a separate experiment, the basal medium was modified to contain 0.1 times the original amounts of amino acids and vitamins. Under these

[5] Thiamine, *p*-aminobenzoic acid, choline chloride, inositol, pyridoxamine, pyridoxal, guanine, uracil, xanthine, norvaline, and ammonium chloride.

conditions, *Lactobacillus casei* ε and *Lactobacillus delbrückii* LD5 showed a definite but partial requirement for asparagine over short incubation periods. A similar, but lesser, effect was observed for about half of the remaining organisms. Since all three cultures (two of *Lactobacillus delbrückii* LD5 and one of *Lactobacillus casei* ε) behaved similarly, it was concluded that aspartic acid (or asparagine) is essential for *Lactobacillus casei* ε only in the absence of uracil.

It was stated by Hegsted (11) in 1944 that "the term 'essential amino acid' may have significance only in relation to the composition of the basal medium." The validity of this conclusion is recognized, although other variables, especially the incubation time, may be of equal or greater importance. Hegsted pointed out that of the eight, nine and ten amino acids found to be essential for *Lactobacillus arabinosus* 17-5, by Shankman

TABLE VII

*Maximum Increase in Acid Production Resulting from Omission of Amino Acid**

Amino acid absent from medium	L citrovorum (797)		L dextranicum (8358)		L delbrückii LD5 (9595)		L casei (7469)	
	Volume NaOH	Time	Volume NaOH	Time	Volume NaOH	Time	Volume NaOH	Time
	ml	hrs.	ml	hrs	ml	hrs	ml.	hrs
Arginine	0.8	52						
Threonine					5.4	50	5 4	75
Glycine					2.9	50	2.4	75
Asparagine	0.5	52	0.5	50	5.7	74	2.4	75
Alanine			1.3	50	0.9	50	1.2	51

* Each volume of NaOH represents the maximum *negative* difference between the volume of 0 01 N NaOH required to titrate 1 ml. of final solution containing the complete medium and that with one amino acid absent.

(10), Kuiken *et al.* (12), and Hegsted (11), respectively, only six amino acids (cystine, glutamic acid, isoleucine, leucine, tryptophane, and valine) were listed as essential by all three authors. It seems significant that, of the six amino acids listed, all but one (cystine) has been found to be required by *Lactobacillus arabinosus* 17-5 on the enriched medium employed in the present experiments, and that this amino acid was shown to be essential in two of the three laboratories referred to by Hegsted. On the other hand, cystine was required on the less complete media, but not on the enriched medium, while arginine and methionine were required on the enriched medium but only on one or two of the less complete media. That microorganisms have such definite amino acid requirements, even on media of widely varying composition, may explain in part why it has been possible to develop satisfactory microbiological assays for the determination of some amino acids in a relatively short time.

The conclusions that "the method of culture of the test organism is a very important factor in the response of the bacteria to the nutrients assayed" and that "the culture method has an important rôle in the production of successful microbiological assays" have been drawn recently by Nymon and Gortner (29) from their study of acid production by *Lactobacillus arabinosus* (8014) and *Lactobacillus casei* (7469). These authors found that the decreased linearity of response with added niacin, observed for cultures of *Lactobacillus arabinosus* transferred every 3 or 4 weeks in yeast extract-dextrose agar, was overcome after a series of transfers through skim milk and subsequent transfers through nutrient-rich liver-tryptone broth. Since these investigations were limited to niacin, it does not follow

TABLE VIII

Rates of Acid Production for Six Cultures of Lactobacillus arabinosus 17-5 (8014)

Culture No [*]	Ml. of acid[†]					
1	2.0 (6)	5.2 (10)	11.0 (24)	12.2 (30)	15.6 (52)	17.0 (74)
2	1.8 (6)	5.3 (10)	11.6 (24)	12 7 (30)	16.2 (52)	16 6 (74)
3	0.8 (6)	3.4 (10)	9.6 (24)	11.1 (30)	14.8 (52)	16 9 (74)
4	1.4 (6)	4.4 (10)	11.2 (24)	12.6 (30)	16 0 (52)	17.0 (74)
5	1.4 (6)	5.2 (10)	10.8 (24)	12.5 (30)	16.3 (52)	17.2 (74)
6	0.7 (6)	3.9 (10)	11.2 (24)	12.5 (30)	15 9 (52)	16.8 (74)

[*] All cultures of *Lactobacillus arabinosus* 17-5 (8014) were obtained from the American Type Culture Collection. The cultures have been maintained for more than 2 years as follows: Nos. 3 and 6 on Bacto-tomato juice agar (Difco), No. 2 on Bacto-proteose-tryptone agar (Difco), and Nos. 1, 4, and 5 on Bacto-yeast-dextrose agar (Difco).

[†] Given as ml. of 0.01 N NaOH to titrate 1 ml. of final solution. The values have been corrected for the blank titration. The figures in parentheses refer to the incubation time in hours.

necessarily that linearity of response would have decreased with other added vitamins or with amino acids.

In the writers' study of this problem, six different cultures of *Lactobacillus arabinosus* 17-5 (8014), obtained initially from the American Type Culture Collection, were carried for more than 2 years by weekly, or bimonthly, transfers on three types of nutrient media. The rates and amounts of acid production by these cultures in the complete basal medium (Table I) are shown in Table VIII. Although the amount of acid produced in 6 hours varied from 0.8 to 2.0 ml., the rates of acid production were such that acid production was nearly uniform from 30 to 74 hours. The maximum drop in acid production by these cultures, with each of the listed amino acids omitted in turn from the basal medium, is shown in Table IX. It may be observed that, under these conditions, the same four amino

acids (glutamic acid, valine, isoleucine, and tryptophane) were essential for all six cultures of *Lactobacillus arabinosus*. There had occurred, therefore, no apparent change in the response to these four amino acids of these six cultures of *Lactobacillus arabinosus* which had been carried as described. The further observation has been made that there has been no significant reduction in the precision and accuracy with which the stated four amino

TABLE IX

Maximum Drop in Acid Production Resulting from Omission of Amino Acid for Six Cultures of Lactobacillus arabinosus 17-5 (8014)*

Amino acid absent from medium	Culture 1		Culture 2		Culture 3		Culture 4		Culture 5		Culture 6	
	Volume NaOH	Time	Volume NaOH	Time	Volume NaOH	Time	Volume NaOH	Time	Volume NaOH	Time	Volume NaOH	Time
	ml	hrs	ml	hrs	ml	hrs	ml	hrs	ml	hrs	ml	hrs.
Glutamic acid	13.6	74	13.4	52	15.7	74	14.3	74	13.3	74	15.8	74
Valine	14.3	74	14.0	52	15.7	74	14.7	74	13.6	52	14.8	74
Isoleucine	13.8	74	13.4	52	15.6	74	14.3	74	13.1	52	15.5	74
Methionine	6.4	52	6.4	52	7.9	74	8.0	52	6.6	52	7.5	52
Arginine	4 1	24	3.6	10	5.8	30	4.1	24	4.0	10	6.1	24
Tryptophane	12.7	74	12.7	52	14.5	74	13.1	74	12.2	52	13.5	74
Cysteine	3.7	30	3.1	24	7.5	24	4.9	24	3.9	24	8.9	24
Histidine	0 6	6	0 6	10	1.3	10	1.1	10	1.6	10	2.1	10
Threonine	2.0	10	2.3	30	2.5	30	1.5	10	2.1	10	2.2	24
Phenylalanine	2.6	10	3 2	24	6.0	52	4.0	30	3.2	10	7.1	52
Tyrosine	1.0	10	1.0	24	1.3	52	1.2	10	1.3	10	1.9	24
Glycine	2.5	10	2 5	10	2.1	10	2.0	10	2.4	10	3.3	24
Asparagine	1.7	10	0 9	10	2.6	30	1.0	10	1.6	10	2.9	24
Lysine	0 3	10	0.2	10	1.0	10	0.4	10	0.7	10	1.8	10
Serine	0.2	6	0.0		0.0		0 3	6	0.5	10	0.8	10
Alanine	0 2	6	0.0		0.2	10	0.2	6	0.6	10	1.1	10
Proline	0.4	6	0.2	6	1.3	10	0 9	10	0.9	10	1.3	10
Norleucine	0 1	6	0.0		0.8	10	0.3	6	0.6	10	1.4	10
Hydroxyproline	0.5	6	0.0		0.6	10	0.2	6	0.5	10	1.5	10
Norvaline	0.1	6	0 0		0.7	10	0.3	10	0.5	10	1.3	10

* See the corresponding foot-notes to Tables V and VIII.

acids could be determined with *Lactobacillus arabinosus* over more than a 2 year period. These results are not wholly in agreement with the conclusions of Nymon and Gortner.

On the other hand, the initial rate of acid production was slowest and the drop in acid production resulting from the omission of some acids (especially arginine, cysteine, methionine, and phenylalanine) from the medium was most marked, with Cultures 3 and 6, which had been carried

on tomato juice agar.[6] Although there were quantitative differences in acid production by the six cultures of *Lactobacillus arabinosus*, the qualitative nutritional requirements were essentially alike. It seems probable, therefore, that the cultures of other organisms (Tables II to VII), which exhibited distinctly different nutritional requirements, represent separate varieties and, possibly, different species. It may be found desirable, therefore, to reclassify some of these organisms.

While it would not be expected that the amino acids for which *Lactobacillus arabinosus* has only partial requirements cannot be determined entirely satisfactorily under the assay conditions so far reported, it seems probable that special conditions might be found under which such amino
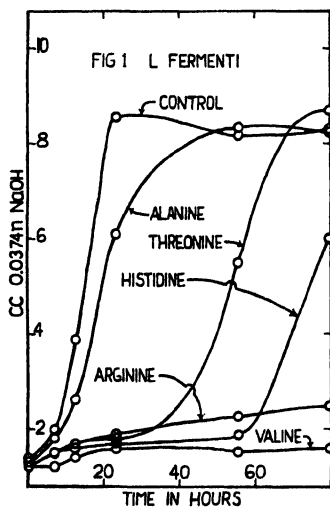
FIG. 1

acids could be determined with reasonable accuracy. The methods suggested recently for the determination of threonine (21) and histidine (30) are considered to be examples of procedures in this category. It was found that these amino acids could be determined satisfactorily after 48 hours incubation, even though threonine was synthesized on the present medium after about 30 hours and histidine after about 50 hours (Fig. 1).

Experiments are now in progress to determine the suitability of some of the microorganisms investigated in the present work for the determination of some of the amino acids.

[6] It is of interest that Kuiken *et al.* (12) carried their cultures of *Lactobacillus arabinosus* on tomato juice agar and that they added a tomato juice preparation to their basal media.

SUMMARY

The response of twenty-three lactic acid bacteria grown in a basal medium of arbitrary composition has been determined. It has been found that acid production was not lowered significantly at levels of total amino acids and vitamins one-fourth those employed initially, that the number of amino acids essential for the microorganisms ranged from two (glutamic acid and valine) for *Leuconostoc mesenteroides* (8293) to fifteen (exclusive of serine, alanine, proline, hydroxyproline, norleucine, and norvaline) for *Lactobacillus brevis* (8257), that certain amino acids were inhibitory for four microorganisms, that of the microorganisms investigated only *Lactobacillus brevis* (8257) required proline (except when excess hydroxyproline was present) over incubation times up to 140 hours, and that there were variations of some significance in the response of six cultures of *Lactobacillus arabinosus* (8014) which had been maintained for more than 2 years on three different culture media. It is considered probable that reasonably satisfactory assay procedures could be developed for each amino acid (except hydroxyproline, norleucine, and norvaline) with from one to twenty-three of the lactic acid bacteria. Experimental studies leading to the possible development of new or improved procedures for the determination of certain amino acids are in progress. Evidence has been presented pointing to the identity of *Lactobacillus delbrückii* LD5 (9595) and *Lactobacillus casei* ε (7469).

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VITAMIN REQUIREMENTS OF TWENTY-THREE LACTIC ACID BACTERIA*

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The investigation of the amino acid requirements of twenty-three lactic acid bacteria, described in the preceding paper (1), was unique in that an exceptionally rich basal medium and a wide range of incubation times were employed in the determination of each amino acid requirement. It seemed desirable to extend this study to other nutritional factors, since there is need for additional information on the nutrition of organisms which might be employed in microbiological assays. This is indicated by the known interrelations between growth factors such as the replacement by alanine of the pyridoxine requirement of *Streptococcus faecalis* R (2), the synthesis of threonine in the presence of pyridoxal by *Lactobacillus arabinosus* 17-5 (3), and the synthesis of aspartic acid in the presence of uracil by *Lactobacillus casei* ε (1). Further nutritional data might also be useful in identifying these organisms, since, for example, it was concluded from the preceding work (1) that *Lactobacillus delbrückii* LD5 had been classified incorrectly. Although the vitamin requirements[1] of numerous lactic acid bacteria have been extensively investigated, systematic studies of the requirements of all of the known vitamins on synthetic media of high nutritional quality and over a wide range of incubation times have not been attempted previously. The vitamin requirements of twenty-three lactic acid bacteria have been investigated in the present experiments.

EXPERIMENTAL

The organisms, the techniques, the experimental conditions, and the basal medium were the same as those given in the preceding paper (1), except that synthetic folic acid,[2] instead of a concentrate of this vitamin,

[1] Comprehensive reviews have been given by Knight (4), Peterson and Peterson (5), and Snell (6, 7).

[2] Obtained from the Lederle Laboratories through the courtesy of Dr. S. M. Hardy. Although the organisms grew as well on the basal medium containing

was used in all of the experiments. The rate of growth of the organisms was determined in terms of acid production. The experimental results are shown in Tables I to IV.

DISCUSSION

The maximum amounts of acid produced by each organism on each type of medium at the indicated incubation times are given in Table I. The vitamins are listed in Table I approximately in order of their nutritional importance, as judged by the results of these experiments. The essential vitamins were considered to be those whose omission from the basal medium

TABLE I

*Maximum Acid Production in Absence of Vitamin**

Vitamin absent from medium	L. mesenteroides (8293)		Leuconostoc citrovorum (7813)		L. citrovorum (797)		L. mesenteroides (9135)		Leuconostoc dextranicum (8086)		L. pentosus (124-2)		Lactobacillus brassicae (8041)		Lactobacillus buchneri	
	ml	hrs	ml	hrs	ml.	hrs	ml	hrs	ml	hrs.	ml.	hrs.	ml.	hrs.	ml.	hrs.
Nicotinic acid	**1.6**	120	**1.3**	119	**1.8**	119	**1.5**	46	**1.5**	117	4.0	164	**3.7**	123	**0.5**	89
Pantothenic acid	**2.1**	120	**1.8**	119	**1.5**	119	**1.7**	120	**1.7**	117	5.9	164	**4.7**	123	**1.3**	160
Folic acid	**4.5**	120	8.4	119	5.1	119	9.6	46	5 0	70	16.4	71	16 3	76	**4.5**	160
Biotin	9.8	71	9.7	46	10.1	46	8.2	71	9.5	70	6.7	164	6.2	123	**1.7**	160
Riboflavin	9.7	47	9.6	29	9.7	46	6.3	120	4.3	117	16.4	71	16.5	76	**0.9**	160
Thiamine .	7.1	120	7.1	70	7.4	119	4.6	71	4.8	117	16.1	71	16.1	76	**1.0**	160
Pyridoxine .	9.5	71	9.6	70	9.7	46	6.2	120	9.5	70	16 1	71	13 4	76	4.3	160
p-Aminobenzoic acid.	9.7	72	10	70	9.1	46	9 7	71	9 3	70	16 4	71	16.7	55	4.7	160
Control	9.9	71	9.9	46	9.9	71	9.8	46	9.1	46	16.1	71	16.0	123	4.6	160

* Acid production given as ml. of 0.01 N NaOH to titrate 1.0 ml. of final solution per tube. The values are corrected for the blank titration. The figures given in bold-faced type are less than one-fourth the control titration. The vitamins corresponding to these figures are considered to be essential.

permitted growth of the organism equivalent to a titration value (given in bold-faced type in Table I) less than approximately one-fourth that found in a control experiment. The number of organisms for which the

crystalline folic acid as on that containing the concentrate, the possibility is not excluded that other forms of folic acid would have greater activity. It is also possible that the growth rate might be increased beyond that recorded in the present paper by the tomato juice eluate of Lyman et al. (8), the strepogenin of Sprince and Woolley (9), the nucleoside of Hutchings et al. (10), the liver and yeast factors of Stokstad (11), the liver and whey supplements of Cooperman et al. (12), the vegetable juice factors of Metcalf et al. (13), and other natural materials. In agreement with the observation of Johnson (14), it was found that nicotinamide was not required by any of the organisms studied in the present experiments.

stated vitamin was required are given in parentheses: nicotinic acid (18), pantothenic acid (17), folic acid (4), biotin (2), riboflavin (3), thiamine (2), pyridoxine (2), and p-aminobenzoic acid (0). The omission of choline or inositol had no effect on the growth of any of the organisms. It seems probable that each of the essential vitamins could be determined satisfactorily by the organisms requiring it, since growth in the absence of an essential vitamin was not significant even after prolonged incubation with relatively heavy inoculation and in the presence of exceptionally high concentrations of other vitamins and nutrients.

The maximum drop in acid production by each organism resulting from the omission of each of the vitamins in turn from the control medium is shown in Table II. The italicized figures represent values which are at least two-thirds the largest drop observed in acid production and it is

TABLE I—*Continued*

Vitamin absent from medium	L. arabinosus 17-5 (8014)		L. citrovorum (8082)		Lactobacillus lycopersici (4005)		L. dextranicum (8358)		L. dextranicum (8359)		Lactobacillus fermentis 36 (9338)		Lactobacillus mannitopoeus		L. delbrückii LD5 (9595)	
	ml	*hrs*	*ml*	*hrs.*	*ml.*	*hrs*	*ml*	*hrs*	*ml*	*hrs*	*ml*	*hrs.*	*ml*	*hrs.*	*ml*	*hrs*
Nicotinic acid	11.3	164	**0.6**	89	**0.6**	92	6 8	70	**1.5**	117	**1.1**	165	0.4	91	**2.1**	69
Pantothenic acid	14.1	164	**0.8**	89	**1.3**	163	**1.7**	117	**1.6**	117	**1.4**	165	**0.6**	91	**1.3**	161
Folic acid	16 1	48	4 8	113	8 5	70	6.7	70	**1.0**	46	8 5	29	8 6	46	7.9	161
Biotin	15 2	164	2 0	113	4 3	163	6.8	70	9 1	46	8.8	49	2 5	46	5.4	161
Riboflavin	16 2	48	2 7	89	2.3	163	5.3	70	8 8	28	8 5	49	8 6	46	**1.3**	161
Thiamine	16 0	48	3 5	89	6 5	163	2 7	70	**4.8**	117	8.2	72	2 2	162	16.0	161
Pyridoxine	16 1	164	2 0	113	8 7	70	6 7	45	9.2	70	8 8	72	8.5	69	**1.6**	161
p-Aminobenzoic acid	16 3	48	5 0	113	8.7	70	6.8	45	9.5	46	8.7	49	8 4	69	16.1	161
Control	16.2	48	4.9	113	8 6	163	7.1	45	9.2	70	8.6	72	8.1	162	15.8	90

considered that successful assays might be made for these vitamins. The figures which are given in bold-faced type indicate those vitamins which are essential for the organisms only under restricted times of incubation. The number of organisms for which the stated vitamins had this type of essentiality are given in parentheses: nicotinic acid (4), pantothenic acid (5), folic acid (3), biotin (9), riboflavin (2), thiamine (5), pyridoxine (0), and p-aminobenzoic acid (0).

The vitamin requirements found in the present work, as well as those reported in the literature, for fourteen of the organisms are summarized in Table III. Although nearly the same vitamin requirements were found in other laboratories, a number of differences may be noted. Some of the vitamins reported in the literature to be essential were found to be nonessential or to be essential only over a short incubation time. That these

vitamins were synthesized under the present experimental conditions was not unexpected, since the synthesis of growth factors including folic acid

TABLE I—*Concluded*

Vitamin absent from medium	L casei (7469)		Lacto-bacillus gayonii (8289)		S. faecalis R (8043)		L citro-vorum (8081)		Lacto-bacillus pento-aceticus (367)		L. mesen-teroides P-60 (8042)		L. brevis (8257)	
	ml	hrs	ml	hrs	ml	hrs.	ml	hrs.	ml.	hrs.	ml	hrs	ml.	hrs.
Nicotinic acid	4.4	162	**1.0**	163	**1.8**	161	2.0	94	**0.5**	163	5.2	117	**0.2**	162
Pantothenic acid	4.1	162	**1.1**	92	**1.9**	161	1.8	94	**1.1**	92	**3.0**	71	**0.9**	91
Folic acid ..	11 0	162	8 6	47	**0.8**	161	2.2	94	**1.3**	71	13 5	117	**0.5**	91
Biotin	8 6	162	8 8	92	**0.4**	161	2.2	94	4.3	163	**2.7**	117	3.3	162
Riboflavin	**2.1**	162	8 3	92	7.7	90	2.4	70	8.1	92	14.6	117	8.4	162
Thiamine ..	16 0	162	4 2	92	7.6	90	2 2	94	2.8	163	13 5	117	**1.5**	162
Pyridoxine	**2.8**	68	8 6	92	6.7	161	2.1	94	8.6	71	10.8	117	8.8	162
p-Aminobenzoic acid	16 0	162	8.4	70	7.7	90	2.2	94	8.5	92	13.9	117	8.6	162
Control . ..	15 7	162	8.5	47	7.8	161	2.3	94	8.2	163	13.4	117	8 4	162

TABLE II

*Maximum Decrease in Acid Production Resulting from Omission of Vitamin**

Vitamin absent from medium	L mesen-teroides (8293)		Leuconostoc citrovorum (7013)		L citrovorum (797)		L mesen-teroides (9135)		Leuconostoc dextranicum (8086)		L pentosus 124-2		Lactobacillus brassicae (8041)		Lactobacillus buchneri	
	ml	hrs	ml	hrs	ml	hrs.	ml	hrs	ml	hrs	ml.	hrs.	ml.	hrs.	ml	hrs.
Nicotinic acid	8 3	47	8 7	46	7.8	29	8 0	46	7.7	28	*13.3*	71	*13.9*	54	*4.3*	160
Pantothenic acid	7 9	47	8.3	46	7.7	29	8.0	46	7.8	28	**11.9**	71	**13.0**	54	**3.4**	160
Folic acid	**6.1**	47	5.2	21	**5.5**	29	0.1	71	**5.6**	28	0.5	48	0.2	31	0.2	160
Biotin	0 8	22	0 3	70	0.8	11	2.0	46	1.8	28	**12.3**	48	**12.0**	54	**3.0**	160
Riboflavin	1.6	11	0.4	70	0.4	11	4.7	46	**7.2**	28	0.2	48	0.3	31	*3.8*	160
Thiamine	2.9	22	3 1	29	2.8	22	4.4	46	**5.4**	28	0.3	48	0.8	31	*3.6*	160
Pyridoxine	0 8	11	−0 2	29	0.7	11	5.2	46	1.9	20	1.4	48	4.1	54	0.1	90
p-Aminobenzoic acid	0.6	22	0 6	21	0.9	22	0.3	46	0.9	46	1.3	28	−0.9	31	0.1	90

* The drop in acid production is given as ml. of 0.01 N NaOH to titrate 1 ml. of final solution per tube   The values given in bold-faced type are at least two-thirds of the maximum drop observed for each particular organism.   It is assumed that the vitamins corresponding to the bold-faced figures might be assayed satisfactorily by the organisms indicated under restricted, but selected, times of incubation. The italicized values correspond to those given in bold-faced type in Table I.

(17), riboflavin (18), and pyridoxine (20) has been observed on the relatively incomplete media employed in other laboratories.

The effect on maximum acid production by the various organisms was investigated with basal media from which either pyridoxine or pyridoxal

and pyridoxamine had been omitted. The significant data are shown in Table IV. As was expected from the experiments of Snell (46, 32), maximum acid production was decreased in the absence of pyridoxal and pyridoxamine, even though the basal medium contained a relatively high concentration of pyridoxine. On the other hand, acid production was nearly maximal in the medium containing pyridoxal and pyridoxamine but no pyridoxine. Pyridoxine, pyridoxal, and pyridoxamine were slightly inhibitory to acid production by *Lactobacillus brevis*.[3] No other example of inhibition by vitamins has been found in the literature.

Although *p*-aminobenzoic acid was not required by any of the organisms investigated in the present experiments, this vitamin has been reported to be essential for *Lactobacillus arabinosus* 17-5 (29–31) and essential under restricted conditions for *Leuconostoc mesenteroides* P-60 (48). Numerous natural materials have been assayed for *p*-aminobenzoic acid with these

TABLE II—*Continued*

Vitamin absent from medium	L arabinosus 17-5 (8014)		L citrovorum (8082)		Lactobacillus lycopersici (4005)		L dextranicum (8358)		L dextranicum (8359)		Lactobacillus fermenti 36 (9338)		Lactobacillus mannitopoeus		L delbrückii LD5 (9595)	
	ml	hrs	ml	hrs	ml	hrs	ml	hrs	ml	hrs	ml	hrs	ml	hrs	ml	hrs
Nicotinic acid	9.1	28	4.5	113	8.5	70	6.0	45	7.6	28	8.0	29	8.5	46	14.2	90
Pantothenic acid	7.1	28	4.1	113	8.0	70	5.8	45	7.4	28	7.6	29	8.1	69	14.8	90
Folic acid	0.2	48	0.3	89	0.3	27	0.3	27	0.1	21	0.3	72	0.8	14	9.9	90
Biotin	8.2	28	2.0	43	6.2	74	1.9	27	1.0	28	2.1	17	6.4	46	10.5	69
Riboflavin	0.1	28	2.0	113	6.5	47	2.3	27	1.3	21	2.1	17	0.1	69	14.8	90
Thiamine	0.6	16	1.3	113	6.4	47	4.4	45	5.4	28	2.6	17	7.0	46	1.4	90
Pyridoxine	5.1	28	1.7	89	0.4	27	0.3	70	0.2	10	2.2	17	1.8	26	14.9	90
p-Aminobenzoic acid	1.0	16	0.2	43	−0.2	92	0.3	10	0.5	10	1.3	17	4.0	26	0.8	90

strains by the procedures described in the papers referred to. The apparent lack of specificity for *p*-aminobenzoic acid of any of the organisms studied in the present work might possibly be accounted for by contamination of the basal medium with this vitamin. It seems more probable, however, that synthesis occurred, since no requirement for *p*-aminobenzoic acid by fourteen of the microorganisms could be demonstrated even at concentrations of vitamins and amino acids one-tenth those present in the unmodified basal medium. Since not more than 0.002 millimicrogram of *p*-aminobenzoic acid per tube could have been carried over from the medium by the inoculum, this source of possible significant contamination was excluded.

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[3] In other experiments inhibition of *Lactobacillus casei* ε and *Lactobacillus delbrückii* LD5 by *p*-aminobenzoic acid was observed during the initial stages of incubation.

On the other hand, the essentiality of p-aminobenzoic acid observed by other workers for the stated organisms, may be restricted to the relatively incomplete basal media employed. This view is supported by the report of Snell and Mitchell (21) that the requirement of *Lactobacillus arabinosus* and *Lactobacillus pentosus* is non-specific for p-aminobenzoic acid, since other nutrients (methionine, adenine, guanine, xanthine, and hypoxanthine) were also effective in promoting growth of these organisms. It was stated that these substances did not contain p-aminobenzoic acid impurity, since treatment with decolorizing carbon and recrystallization did not change their activity. The probability that the basal media, utilized by the authors quoted for the determination of p-aminobenzoic acid, may have been somewhat incomplete in respect to amino acids is indicated by the

TABLE II—*Concluded*

Vitamin absent from medium	L casei (7469)		Lacto-bacillus gayonsi (8289)		S faecalis R (8043)		L citro-vorum (8081)		Lacto-bacillus pento-aceticus (367)		L mesen-teroides P-60 (8042)		L. brevis (8257)	
	ml	*hrs.*	*ml*	*hrs.*	*ml*	*hrs*	*ml*	*hrs.*	*ml*	*hrs*	*ml.*	*hrs*	*ml*	*hrs*
Nicotinic acid	12.7	90	8.3	47	6.4	90	0.4	70	7.8	92	9.5	117	8.3	91
Pantothenic acid	13.4	90	7.9	47	6 5	90	0.4	94	7.1	71	12.1	117	7.5	91
Folic acid.	8.0	90	0.6	27	7.0	90	0.0	94	7.3	92	1.2	117	8.3	162
Biotin.	10.4	90	4.6	47	7.4	90	0	94	4.8	71	10.5	71	5.9	91
Riboflavin	14 0	90	1.9	27	−0.2	45	0	94	0.2	71	0.5	47	0.4	91
Thiamine	1.0	69	5.3	47	0.3	45	0	94	6.0	71	1.2	117	7.2	91
Pyridoxine .. .	14.4	90	2 5	47	1.9	68	0	94	−0.9	71	4.0	71	−0.2	91
p-Aminobenzoic acid	0.6	69	2.6	92	−0.2	45	0	94	0.8	71	0.8	71	0.4	26

writers' observation (unpublished data) that significant amounts of several amino acids, and marked quantities of some others (especially phenylalanine and tyrosine), were removed from an acid hydrolysate of casein which had been treated with decolorizing carbon. The further possibility was suggested by Snell (quoted by Shankman (50)) that there are two strains of *Lactobacillus arabinosus* 17-5 of which one, a mutant strain, does not require p-aminobenzoic acid. Lewis has stated (quoted by Pennington (48)) in this connection that "*Lactobacillus arabinosus* 17-5 could be trained to give relatively stable p-aminobenzoic acid nonrequiring lines by subculturing in the presence of suboptimal levels of p-aminobenzoic acid" and that "recently his stock culture gained the ability to dispense with p-aminobenzoic acid after maintaining a stable requirement for at least three years."

TABLE III

*Vitamin Requirements of Fourteen Lactic Acid Bacteria from Present Work and Literature Reports**

Organism	Nicotinic acid		Pantothenic acid		Folic acid		Biotin		Riboflavin		Thiamine		Pyridoxine		p-Aminobenzoic acid	
L. pentosus	E		E'	E (15, 16)	N	N (17)	E'		N	N (18, 19)	N		N	N (20)	N	N (21)
Lactobacillus brassicae	"		"	" (15)	"	" (17)	"		"	" (18)	"		S		"	
Lactobacillus buchneri	"		"		"	" (26)	"		E	E (22)	E	E (22)	N		"	
L. arabinosus	E'	E (23, 24)	"	" (15, 25)	"		"	E (25, 27)	N	N (25, 19)	N	N (25), E' (28)	S	N (25, 20)	"	N (21), E (29–31), S (32)
Lactobacillus lyco-persici	E		E	E (16)	"		"		E'	E (22)	E'	E (22)	N		"	
Lactobacillus fermenti 36	"		"	" (33)	"		N		N	N	N	" (34)	"		S	
Lactobacillus manni-topoeus	"		"	" (15, 16)	N		E'		"	N (18, 22)	E'	" (22)	"		N	
L. delbrückii LD5	"	E (35)	"	" (35)	S	E (35, 36)	"	E (35)	E	E (35)	N		E	E (35)	"	
L. casei	E'	E (23, 24)	E'	" (15, 37)	"	" (38–40)	"	E (27, 41)	"	" (18, 19)	"	E' (28)	"	" (38, 42)	"	N (31), S (43)
Lactobacillus gayoni	E	E (24)	E	E (15)	N	N (17)	S		N	" (18)	S		N		"	
S. faecalis R	"		"	" (15)	E	E (17, 42)	E	E (27)	"	N (18)	N		"	" (44–46), N (2)	"	
Lactobacillus pento-aceticus	"		"	" (15)	"	N (17)	S		"	" (18)	E'		"		"	
L. mesenteroides P-60 (8042)	E'	E (24, 15)	"	" (47, 15)	N	" (47)	E	E (47)	"	" (47, 18, 19)	N	E (47)	S	E (47)	"	E' (48)
L. mesenteroides (9135)	E	E (49)	"	" (49)	"		S	" (49)	S	" (49)	S	" (49)	"	S (49)	"	

* The vitamin requirements of the nine additional organisms are indicated in Tables I and II. The symbols given in the first column under the name of each vitamin refer to requirements found in the writers' laboratory, and those in the second column to the requirements reported in the literature. The numbers given in parentheses refer to bibliographic citations. The symbols have

TABLE IV

Effect of Pyridoxine, Pyridoxal, and Pyridoxamine on Maximum Acid* Production by Lactic Acid Bacteria

Organism	Incubation time	Vitamin omitted			
		Pyridoxine, pyridoxal, pyridoxamine	Pyridoxal, pyridoxamine	Pyridoxine	None
	hrs.	ml.	ml.	ml.	ml
Lactobacillus brassicae (8041)	123	14.50	15.13	17.30	17.10
L. arabinosus (8014)	28	10.50	11.34	15.10	15.60
L. casei (7469)	161	3.20	9.60	14 00	16.80
Leuconostoc citrovorum (8082)	113	3 50	4.21	5.49	6.20
L. delbruckii (9595)	161	2.60	5.72	8.70	16.90
L. mesenteroides (8042)	163		10.22	15.02	15.88
L. mesenteroides (9135)	120	7.70	8.50	10.70	10.81
L. brevis (8257)	69	8.14	8.00	7.50	7.50

* Given in terms of ml. of 0 0206 N NaOH to titrate acid in 2 ml. of final volume solution per tube.

SUMMARY

The vitamin requirements of twenty-three lactic acid bacteria have been studied over a wide range of incubation times on a completely synthetic medium which was exceptionally rich in nutrients. The experimental results have been discussed in relation to nutrition studies which have been reported in the literature. It was considered probable that satisfactory assay procedures could be devised for the determination of pyridoxine, thiamine, biotin, riboflavin, folic acid, pantothenic acid, and nicotinic acid with from two to eighteen organisms. Choline, inositol, and *p*-amino-benzoic acid were not required by any of the strains. The inhibitory effect of pyridoxine and *p*-aminobenzoic acid on acid production by some microorganisms has not been previously reported for these or other vitamins.

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THE EFFECT OF CARBOHYDRATES ON ACID PRODUCTION BY TWENTY-FOUR LACTIC ACID BACTERIA*

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It was observed in the two preceding investigations (1, 2) on the amino acid and vitamin requirements of twenty-three strains of lactic acid bacteria that *Lactobacillus plantarum*, *Lactobacillus acidophilus*, and *Lactobacillus buchneri* grew poorly, if at all, and produced little, or no, acid under the stated experimental conditions. Although the remaining organisms grew satisfactorily and produced relatively large amounts of acid, further studies on the influence of nutrients other than vitamins and amino acids were undertaken. The object of this work was to determine, as far as possible, the types and proportions of nutrients required in the basal media for the most rapid and abundant acid production. It was assumed that the effectiveness of any stimulatory and inhibitory materials, introduced during an assay, would be reduced somewhat in proportion to the improvement in nutritional quality of the basal medium. The effect of carbohydrates on acid production was emphasized in these studies.

Glucose has been employed almost exclusively as the carbohydrate component of media utilized for the determination of vitamins and amino acids, since the first procedure for the quantitative determination of a vitamin (riboflavin) with a lactic acid bacillus (*Lactobacillus casei* ε) was described by Strong and Snell (3) in 1939. In view of its ready fermentation by most lactic acid bacteria, glucose appeared to be superior to most, if not all, carbohydrates for this purpose. There is some evidence in the literature, however, which indicates that certain carbohydrates may be more effective than glucose as the source of acid in media used for the determination of some vitamins and amino acids by assay with particular strains of lactic acid bacteria. It was reported by Fred *et al.* (4) in 1919 that cultures of organisms (named *Lactobacillus pentoaceticus*) isolated from manure, silage, and sauerkraut, and incubated for 2 weeks at 30–35°, gave yields of total volatile acids from xylose[1] which were from 2 to 3 times

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[1] It is of interest that a basal medium containing 1 gm. of xylose and 20 gm. of glucose per liter has been used by Barton-Wright (5) for the determination of tryptophane by assay with *Lactobacillus arabinosus* 17-5.

as great as those obtained from fructose, glucose, sucrose, and lactose. The yields of non-volatile acids were nearly equivalent to those of the volatile acids.

In later studies of the fermentation of pentoses (xylose and arabinose) and other carbohydrates (glucose, fructose, galactose, mannose, sucrose, lactose, maltose, melezitose, and raffinose) by Fred *et al.* (6), it was found that acetic and lactic acids equivalent to about 90 per cent of the sugar destroyed, and approximately the same quantities of total acid, were formed by some of the cultures of lactic acid bacteria (*Lactobacillus pentosus, Lactobacillus arabinosus*, and *Lactobacillus pentoaceticus* types). Since acid production by some of the lactic acid bacteria varies with the type of carbohydrate employed in the basal medium, it would be expected that the precision and accuracy of microbiological assays for vitamins and amino acids might be affected if a carbohydrate different from that present in the basal medium were introduced with the sample of material to be assayed. It appeared desirable, therefore, to study the relation between various carbohydrates and acid production by different strains of lactic acid bacteria. No systematic investigation of this type has come to the authors' attention. The effect of sodium chloride has been determined in comparable studies, since this salt invariably is present in hydrochloric acid hydrolysates of protein materials. In addition, the possible stimulatory action of a series of natural materials with which bacteriological media are commonly supplemented was tested.

EXPERIMENTAL

The basal medium, the experimental conditions and techniques, and the organisms were the same as those employed in preceding investigations (1, 2), except for the following modifications. Ammonium chloride, sodium chloride, xanthine, and all of the amino acids were omitted from the basal medium, and 15 gm. of Bacto-peptone (Difco) were added per liter of medium. The inoculum was grown on the modified basal medium and each tube was inoculated with 1 drop of the culture. The glucose in each basal medium employed in the fermentation experiments was replaced by an equal weight of one of the carbohydrates given in Table I, and an inverted glass vial was placed in each tube for the collection of gas. The carbohydrate test media were sterilized by steaming for $\frac{1}{2}$ hour at 100°, instead of by autoclaving, and 0.05 per cent of caramelized glucose (prepared by autoclaving a mixture containing 1 gm. of glucose, 0.6 gm. of sodium acetate, 0.5 gm. of peptone, and 10 ml. of water) was added to the medium as a source of intermediate compounds reported to be essential for initiation of fermentation by some organisms (7, 8). The incubation

time was 72 hours in all experiments, except where otherwise stated, and 3 ml. volumes of medium were employed in 4 inch, soft glass test-tubes.

The experimental results, given in Tables I to IV, represent the averages of duplicate determinations.

DISCUSSION

The ml. of 0.01 N acid produced in 72 hours by twenty-four lactic acid bacteria on twenty-two media, each containing 2 per cent concentration of a particular carbohydrate, are shown in Table I. Gas (indicated in the table) was formed in about 10 per cent of the 528 test samples. The number of carbohydrates (and carbohydrate derivatives) from which gas was produced by the various organisms ranged from eight for *Leuconostoc mesenteroides* (8293) to one for *Lactobacillus buchneri*. No gas was formed by ten organisms on any of the media. The number of organisms which produced gas on the various carbohydrates ranged from twelve for maltose to two for lactose. No gas was formed from thirteen of the carbohydrates. Organisms which produced acid readily are considered to be those for which acid production was more that two-thirds (indicated as bold-faced figures in Table I) that formed on the glucose-containing medium. The numbers of organisms which produced acid readily are given in parentheses: glucose (23), fructose (21), maltose (19), trehalose (13), galactose (12), arabinose (11), salicin (8), sucrose (9), lactose (7), xylose (7), raffinose (6), and mannitol (5). The number of carbohydrates from which acid was produced readily by the various organisms ranged from twelve for *Lactobacillus arabinosus* (8014) to two for *Leuconostoc dextranicum* (8359).

It is of interest that one of the most fastidious organisms, *Lactobacillus buchneri*, produced very little acid in the present as well as in previous (1, 2) experiments on media containing glucose, whereas abundant acid was formed from xylose and from arabinose. A significant amount of acid was produced from fructose but not from any of the remaining carbohydrate forms. It is evident that *Lactobacillus buchneri* might be employed for assay purposes with a basal medium containing either xylose or arabinose.

Although none of the other organisms was as specific as *Lactobacillus buchneri* in its carbohydrate requirements, many strains produced more acid from other sugar substances than from glucose. Relatively high acid production was most common with the pentoses (xylose and arabinose). Five strains yielded considerably more acid from xylose, and six from arabinose, than from glucose. On the other hand, xylose was fermented poorly, and arabinose readily, by several strains, while the opposite fermentation tendencies were exhibited by several other strains. It may be

noted, also, that acid production by *Lactobacillus lycopersici* and by *Lactobacillus brevis* was unusually high both on xylose and arabinose. Seven carbohydrates were readily fermented by *Lactobacillus fermenti* 36 (9338), but xylose yielded approximately twice as much acid as any of the other sugars, including glucose. It would seem probable, therefore, that assays of samples containing relatively large amounts of xylose might not be entirely satisfactory with a glucose-containing basal medium. Conversely, the quality of assays with this organism might be improved by the

TABLE I

Acid Production*

Carbohydrate or carbohydrate derivative	L mes-enter-oides (8293)	L cit-rovor-um (7013)	L cit-rovor-um (797)	L. mes-enter-oides (9135)	L dex-trans-cum (8086)	L. pent-osus 124-2	L brass-icae (8041)	L. buch-ners†
Glucose.	9.3‡	10.3‡	9.7‡	10.3‡	8.9‡	16.5	15.9	0 4
Fructose. .	7.0‡	6.8‡	6.6	7.4	8.2‡	15.0	14.8	6.4‡
Mannose	4.9	5 3	6.1	1 3	6.9‡	16.3	17.1	0.1
Galactose . . .	5.6‡	5.2	4.8‡	1.8	3.0	15.0	15.8	0 8
Xylose .	4.2	4 9	3 8	3.8	1.8	8.9	6.3	17.6
Arabinose	4.7	4.6	3 9	6.1	1 3	12.3	7.1	21.7
Rhamnose .	0.6	0 6	0.6	0.6	0 4	2.6	1.4	0.0
Salicin .	1 7	3 5	4.1	4.5	0 1	13.1	11.5	0.0
Maltose...	9.1‡	9.5‡	9.1‡	2.1	7.1‡	15.7	15.5	0.3
Sucrose. ..	6.1‡	6.1‡	5.9‡	7.5‡	7.3‡	16.6	16.3	1.5
Lactose ..	5.9‡	4.3	4.1	1 5	1.7	14.2	14.9	0 0
Trehalose	8.8‡	8.9‡	8.7‡	9.5‡	5.9‡	16.9	16.7	0.0
Raffinose	6 3‡	6 2‡	5.0‡	1.2	1.2	14.8	1.2	0.2
Dextrin.. . .	0.1	1.7	1.5	0.1	0.3	1.9	1.9	0.1
Amylose . .	0 0	0.2	0.2	0.3	0.2	0.6	0.5	0.0
Glycogen . . .	0.4	0 6	0.4	0.6	0.8	1 0	1.0	0.0
Inulin	0.4	0.6	0.6	0.4	0.6	0.6	0.7	0.0
Mannitol	1.5	2.1	2.9	1.3	0.3	11.3	11.5	0.1
Dulcitol. . . .	0.4	0.6	0.6	0.4	0.4	1.0	1.0	0.1
Sorbitol	0 6	1.0	1.0	0.8	0.6	1.0	1.3	0.2
Inositol. .	1.0	1.0	1.0	2.6	0.4	1.2	1.2	0.2
Glycerol . . .	0.9	1.2	1.3	0.8	0 6	5.4	5.3	0.2

use of a xylose-containing basal medium. Consideration of the carbohydrate preferences of *Lactobacillus fermenti* 36 is of interest since procedures have been reported for the determination of thiamine (9), histidine (10), threonine (11), and methionine (12) with this organism. Acid production was not increased significantly on media containing carbohydrates, other than xylose and arabinose, in place of glucose.

The fermentation characteristics (Table I) of some of the organisms are atypical, according to the descriptions of these species given in Bergey's

(14) manual.   Examples of *Leuconostoc* strains which appear to be in this category are *citrovorum* (7013), *citrovorum* (797), and *dextranicum* (8358), which, probably, should be classified as strains of *Leuconostoc mesenteroides* because of their ability to ferment sucrose and pentoses.   Another *Leuconostoc* strain, *mesenteroides* P-60 (8042), probably should be classified as *Leuconostoc citrovorum*, since it does not ferment sucrose.   Other examples may be cited from the genus *Lactobacillus*, although complete classification could not be made on the basis of carbohydrate reactions alone.   It ap-

TABLE I—*Continued*

Carbohydrate or carbohydrate derivative	L arabinosus 17-5 (8014)	L citrovorum (8082)	L lycopersici (4005)	L. dextranicum (8358)	L dextranicum (8359)	L fermenti 36 (9338)	L. mannitopoeus†,§	L. delbrücksii (9595)
Glucose . .	16.5	5.3	9.3‡	9.4‡	10.3‡	9.5‡	9.3‡	16.3
Fructose	15.0	5.2	6.1	7.1	6.6	6.2	6.0	16.6
Mannose .	16.9	5.3	0.1	3.3	3.8	9.1‡	9.3‡	17.5
Galactose	14.4	2.4	9.2‡	1.6	1.4	9.4‡	9.0	16.2
Xylose .	1.4	0.8	22.4	0.8	0.8	16.4	0.4	1.2
Arabinose	11.3	0.5	21.9	6.5	0.9	0.3	18.1	1.3
Rhamnose	1.6	0.2	0.0	0.4	0.4	0 0	0.0	6.4
Salicin	12.1	4.7	0.0	0.4	3.7	0.0	0.0	11.1
Maltose	15.8	4.3	9.1‡	8.8‡	9.7‡	9.4‡	8.9‡	1.5
Sucrose	15.1	0 1	8.7‡	7.3‡	6.1‡	0.0	7.3‡	0.9
Lactose	15.7	1 9	0 1	0.5	0 4	7.9‡	2.9	16.3
Trehalose	15.5	1 5	0.0	4.5	3.1	0 7	0.0	18.1
Raffinose	14.0	1 2	7.2	6.6	0.6	4.4	6.6	1.0
Dextrin	1 7	1 3	0.0	0.5	0.5	0 5	0.1	0.9
Amylose	0.4	0 1	0.0	0.0	0.0	0.4	0.0	0.2
Glycogen	1.0	0.2	0.0	0.4	0.4	0 0	0.0	0.8
Inulin.	0 8	0.2	0 0	0.4	0.4	0.0	0.0	0.8
Mannitol .	12.5	1.3	1.5	3.5	1.9	0.0	0.0	11.9
Dulcitol	1.0	0.1	0 0	0.4	0.4	0.0	0.0	0.6
Sorbitol . .	9.2	0.4	0.0	0 6	0.8	0.0	0.0	3.8
Inositol .	0.6	0.2	0.0	0.4	0 4	0.2	0.0	1.0
Glycerol. . .	1.2	1.2	0.0	1.0	0.6	0.2	0.2	1.0

pears, however, that *Lactobacillus delbrückii* LD5 (9595) is classified incorrectly, since it fermented lactose.   The other characteristics of fermentation, as well as the vitamin (1) and amino acid (2) requirements of this organism, indicate that it is a strain of *Lactobacillus casei* nearly, if not entirely, identical to *Lactobacillus casei* ε (7469).   It may be desirable, also, to give *Lactobacillus buchneri* a special classification on the basis of its inability to ferment glucose.

As shown in Table II, all of the organisms were strongly inhibited by

concentrations of sodium chloride ranging from 1 to 5 per cent and none of the strains was able to grow or produce acid in from 9 up to 14 per cent salt concentration. After an initial period of nearly constant acid production the latter gradually decreased with increasing concentrations of sodium chloride. Acid production by *Lactobacillus fermenti* (9338), *Lactobacillus*

TABLE I—*Concluded*

Carbohydrate or carbohydrate derivative	L. casei (7469)	L. gayonis§ (8289)	Strepto-coccus faecalis R (8043)	L. citrovorum (8081)	L. pentoacetiscus (367)	L mesenterosides P-60 (8042)	L. brevis (8257)	L. plantarum (8008)
Glucose	17.9	9.3‡	7.7	11.1	9.4‡	15.1	9.3‡	9.9
Fructose	16.0	6.3	6.2	12.2	6.8	12.6	6.0‡	9.2
Mannose	17.1	9.3‡	6.3	12.3	0.3	14.5	0.0	10.5
Galactose	16.0	7.2‡	1.4	8.8	3.4	10.2	6.6‡	4.6
Xylose	1.4	17.4	0 8	1.0	9.2	15.4	22.4	1.6
Arabinose	1.5	0.3	0 7	16.5	18.9	17.5	21.7	6.7
Rhamnose	6.4	0.0	0.2	2.4	0.0	2.8	0.0	0.8
Salicin	12.9	0.0	3.9	10.7	0.0	3.3	0.0	6.5
Maltose	1.9	9.1‡	5.9	11.7	8.9‡	0.0	8.1‡	11.3
Sucrose	0.7	0.0	2 9	0.0	0.0	0.0	0 0	7.0
Lactose	15.7	0 0	3 3	2 7	2.5	0 3	0.3	6.5
Trehalose	16.7	0.0	2 3	12.1	0.0	12.7	0.0	9.7
Raffinose	1.0	0.0	0 6	0 2	0 0	0.2	0 0	9.0
Dextrin	0.9	0 3	0 7	0.1	0.0	0.0	0 0	4.7
Amylose	1.0	0.2	0 0	0 0	0.0	0 0	0.0	0.9
Glycogen	1.0	0 0	0.2	0 2	0.0	0.4	0.0	1.2
Inulin	0.8	0 0	0 0	0.0	0 0	0.0	0.0	0 6
Mannitol	12.3	0 0	0.0	0.0	0.9	0.0	1.1	0.7
Dulcitol	0.9	0.0	0.0	0 1	0.1	0.1	0.0	0.9
Sorbitol	3.6	0.0	0.2	0 2	0.0	0.2	0.0	1 0
Inositol	1.0	0 2	0 0	0 0	0 0	0.4	0.2	0 8
Glycerol	0.6	0 0	0 2	0 6	0 0	0 4	0.0	1.2

* Acid production is given as ml. of 0.01 N NaOH to titrate 1 ml of final solution per tube containing 2 per cent concentration of the stated carbohydrate. Except for *L. buchneri* the values given in bold-faced type are at least two-thirds those found for glucose.

† Culture obtained through the courtesy of Dr. V. H. Cheldelin.

‡ Gas formed.

§ Similar results for acid production from fourteen carbohydrates by this (or a related) strain has been reported by Stiles *et al.* (13).

mannitopoeus, Lactobacillus lycopersici (4005), and *Lactobacillus gayonii* (8289) was unaffected, or slightly stimulated, by increasing concentrations up to a critical level (2 to 4 per cent) of salt, beyond which it decreased sharply. Comparable similarity in the behavior of these organisms was observed in the experiments on vitamin (1) and amino acid requirements

(2). It seems apparent that these organisms represent species which are closely related, but which may be readily differentiated by fermentation tests with sucrose, lactose, raffinose, xylose, and arabinose (see Table I). Since all of the organisms were inhibited significantly by salt at particular concentrations, it seems probable that they might be inhibited even more on media containing concentrations of an essential nutrient approaching

TABLE II

*Per Cent Salt Tolerance**

Organism	NaCl in basal medium								
	0	1	2	3	4	5	6	7	8
L. mesenteroides (8293) ..	8.8		9.2	6.8	4.4	2.8	1.6	0.2	0.0
L. citrovorum (7013) . .		8 7	9 3	6.4	4.4	2.9	1.7	0.4	0.0
L. citrovorum (797) ..	8.9	9.0	9.2	6 6	4 5	2.8	1.8	1.2	0.0
L. mesenteroides (9135)	9 2	9.1	9.3	8 1	5.4	3.0	0.2	0.0	0.0
L. dextranicum (8086) .	8.2	9.1	8.9	6.1	4.0	1.8	0.2	0.2	0.0
L. pentosus 124-2 . .	14.1	13 9	13.4	12.0	11.0		5.5	2.5	0.8
Lactobacillus brassicae (8041) .. .	14 4	14.5	13.6	12.8	10.8	8.6	4.6	2.3	1.2
L. arabinosus (8014)	15 2	15.5	15.9	14.8	13.0	9.3	4.4	1.4	0.4
L. citrovorum (8082)	5.8	5 6	5 0	4 3	3.2	1.0	0.2	0.0	0.0
L. lycopersici (4005)	8.5	8 6	8.7	8.8	8.8	1.3	0.9	0.2	0.0
L. dextranicum (8358)	5.8	5.2	3.9	3 6	3.0	2.2	1.1	0.2	0.0
L. dextranicum (8359)	6.2	4.4	3 4	2.6	2.0	1.4	0.4	0.2	0.0
L. fermenti (9338) .	8.4	8 3	8.4	8.6	6.2	0.1	0.2	0.0	0.0
L. mannitopoeus .	8 1	8.1	8 0	1.4	0 1	0.0	0.0	0.0	0.0
L. delbrückii (9595) . .	15.4	15 5	14.8	13.3	10.6	4.7	0.1	0.0	0.0
L. casei (7469) .	15.0	14 9	14.2	13.5	12.0	9.0	1.9	0.0	0.0
L. gayonii (8289) .	8.2	8 3	8.5	8.5	8.0	0.4	0.2	0.2	0.0
Streptococcus faecalis R (8043)	5.3	5.7	5.4	4 6	3.4	1.9	0.6	0.2	0.0
L. citrovorum (8081)	11.4	10.1	8 9	7.8	6.8	5.2	3.2	1.4	0.4
L. pentoaceticus (367) .	4.9	5.3	3.6	3.0	1 2	0.6	0.4	0.2	0.1
L. mesenteroides (8042)	10.8	9.6	8 1	6.7	5.6	3.4	1.2	0.0	0.0
L. brevis (8257). . .	8 2	8 4	8 2	8 0	5.6	3 0	1.6	0.4	0.0

* Given as ml. of 0.01 N NaOH to titrate the acid formed per ml. of final solution. No acid was produced by any of the organisms at any concentration of salt from 9 to 14 per cent.

the limiting amounts. It seems evident, therefore, that possible inhibitions by salt should be avoided in assaying materials by microbiological methods. This may be accomplished by eliminating the salt from the sample to be assayed or, as has been described in recent papers (10–12, 15), by adjusting all solutions employed in an assay to the same salt concentration.

Lactobacillus plantarum grew well on the present basal medium but not

on that employed in previous experiments (1, 2). It was concluded, therefore, tha⎣ Bacto-peptone (Difco) contained a growth factor (or factors) in addition to the stated twenty-one amino acids, twelve vitamins, eight salts, three purines, and one pyrimidine. It was found, however, that this accessory material was not present in all lots of Bacto-peptone. For this reason, a series of supplements was tested. The results of these experiments are shown in Table III.

Autolyzed yeast and Bacto-beef were found to be the best sources of the factor. Although liver concentrate powder stimulated the growth of *Lactobacillus plantarum*, the concentration of the factor in this product appeared to be less than one-third that in the other supplements. The other materials were without appreciable stimulatory effect in the quantities employed in these experiments. It seems probable that this *Lacto-*

TABLE III

Acid Production* by Lactobacillus plantarum (8008) on Basal Media Containing Nutrient Supplements

Supplement added per ml of medium	Bacto-peptone† (Difco), No 382113	Yeast extract (Difco), No 369332	Peptonized milk (Difco), No 359304	Water-soluble milk concentrate (Labco), No XXX	Autolyzed yeast (Difco), No 374818	Liver concentrate powder 1 20 (Wilson), No 59265	Bacto-beef (Difco), No 379586
mg.	*ml*	*ml*	*ml*	*ml*	*ml*	*ml*	*ml*
0.33	0 1	0.2	0 3	0 4	14.7	0.6	14.0
1	0 2	0 3	0 3	0 4	16 1	11 0	14.4
5	0.2	0.5	0 7	0 7	17 3	15.9	15 5

* Given as ml. of 0.01 N NaOH to titrate 1 ml of final solution

† The amounts indicated were in addition to the 15 mg of Bacto-peptone present per ml. of basal medium

bacillus plantarum factor is not thiamine, pyridoxine, pyridoxamine, pyridoxal, pantothenic acid, riboflavin, nicotinic acid, biotin, *p*-amino-benzoic acid, folic acid, choline, or inositol, since these vitamins were present in the medium on which no growth occurred. It appears likely that this factor is not coenzyme I, since yeast extract, a good source of this substance, did not support growth of *Lactobacillus plantarum*. The isolation of this factor and the development of an assay method for its determination are under investigation.

The supplementary nutrients, except Labco XXX water-soluble milk concentrate, were tested at a concentration of 5 mg. per ml. of medium to determine their effect on 24 hour acid production by the lactic acid bacteria. The results of these experiments are given in Table IV for twelve of the organisms for which significant stimulation of acid production was ob-

served. The number of organisms in this category ranged from nine for yeast extract to four for Bacto-beef. Since increased acid was formed on Bacto-beef by only four of the twelve organisms, it appears probable that the yeast, milk, and liver supplements contained some growth factor (or factors) in addition to the *Lactobacillus plantarum* factor present in Bacto-beef. It is of interest that Metcalf *et al.* (16) have reported the presence of an unidentified growth-promoting substance (called T factor for *Lactobacillus fermenti* (L36)) in tomato juice and other vegetables. That the T factor is distinct from thiamine was indicated by its stability to heat. None of about forty other substances (vitamins, amino acids, purines, pyrimidines, organic acids principally) enhanced the growth of this or-

TABLE IV

Acid Production* by Lactic Acid Bacteria on Basal Media Containing Nutrient Supplements

Supplement added (5 mg per ml of medium)	L dextranicum (8086)	Lactobacillus brassicae (8041)	L citrovorum (8082)	L lycopersici (4005)	L dextranicum (8358)	L delbrückii (9595)	L casei (7469)	Streptococcus faecalis R (8043)	L citrovorum (8081)	L pentoaceticus (367)	L brevis (8257)	L plantarum (8008)
None	7 6	14 1	2 8	1 9	7 8	12 0	11 8	4 9	11 8	3 1	4 4	0.2
Yeast extract	**9.0**	15 4	**3.2**	**3.3**	8 4	**17.3**	**17.0**	**6.1**	**13.6**	**3.5**	**5.3**	0.4
Peptonized milk	8 0	14 6	**3.5**	**3.9**	8 2	**15.5**	**15.4**	**5.8**	12 6	**4.8**	**5.4**	0 3
Autolyzed yeast	8 3	**15.7**	**3.4**	1 4	**8.6**	**14.5**	**14.3**	4 0	**13.2**	2.4	**5.3**	**3.7**
Liver powder	8 0	14 6	2 7	1 3	7 8	**13.2**	**13.0**	4 8	12 3	**4.7**	4.6	**2.7**
Bacto-beef	**9.0**	14.5	2 8	**2.3**	**8.6**	12 4	12 0	3 9	12.1	2.9	4.8	**2.9**

* Given as ml of 0 01 N NaOH to titrate 1 ml of final solution per tube. The values given in bold-faced type are at least 10 per cent higher than those obtained when no supplement was added

ganism. It seems possible, from the present experiments, that the T factor might be xylose, since it alone, of all the nutrient substances tested, gave a significant increase in acid production (from about 9 ml. of 0.01 N NaOH per ml. of basal medium to about 16 ml.). No report has been found of the presence of xylose in tomatoes, although total sugars average about 3.4 per cent, according to Chatfield and Adams (17).

SUMMARY

It has been found from tests of twenty-four lactic acid bacteria and twenty-two carbohydrates (and carbohydrate derivatives) that the number of carbohydrates from which gas was produced ranged from eight for *Leuconostoc mesenteroides* (8293) to zero for ten organisms, that the number

of organisms which produced gas ranged from twelve for maltose to zero for thirteen carbohydrates, that the number of organisms which produced relatively large amounts of acid ranged from twenty-three for glucose to zero for nine carbohydrates, and that the number of carbohydrates from which acid was produced readily by the various organisms ranged from twelve for *Lactobacillus arabinosus* (8014) to two for *Leuconostoc dextranicum* (8359). Arabinose and xylose were the only carbohydrates which supported good growth and abundant acid production by *Lactobacillus buchneri*. Sodium chloride was well tolerated by all of the organisms up to concentrations which, in some instances, were as high as 4 per cent. Acid production was inhibited at higher salt concentrations, and no acid was formed by any organism beyond 8 and up to 14 per cent concentration of sodium chloride in the basal medium. It was considered probable, from experiments on the stimulation of acid production, that a growth factor essential for *Lactobacillus plantarum* (8008) and stimulatory substances for eleven other organisms were present in nutrient supplements. Some evidence was presented which indicated that *Lactobacillus delbrückii* LD5 (9595), and possibly some other organisms, probably should be reclassified. The possible significance of the present experimental findings to the microbiological assay of vitamins and amino acids has been discussed.

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THE DETERMINATION OF $d(-)$- AND $l(+)$-GLUTAMIC ACID IN PROTEIN HYDROLYSATES*

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The determination of $d(-)$- and $l(+)$-amino acids is of interest because of recent reports that both isomers may be present in normal and tumor tissues, antibiotics, and microorganisms, and that they may follow different pathways in metabolism. Although amino acid antipodes may be estimated by the isotope dilution procedure and by means of specific amino acid oxidases, it would appear advantageous if each optical form could be determined microbiologically.

It may be possible to determine $d(+)$- and $l(-)$-methionine in the presence of each other, since Dunn *et al.* (2) have shown that total $d(+)$- and $l(-)$-methionine may be determined with *Lactobacillus fermenti* 36 and $l(-)$-methionine with *Leuconostoc mesenteroides* P-60 or *Lactobacillus arabinosus* 17-5. The proportions of $d(-)$- and $l(+)$-glutamic acid in bacterial cells were estimated by Camien *et al.* (3), who compared the acid produced at various levels of the hydrolysates with that observed in media containing from 10:1 to 5:3 ratios of the antipodes. Quantitative values were not obtained and it would be difficult to determine the glutamic acid antipodes accurately by this procedure, since the activities of the isomers vary with the concentration (4–7) and a series of standard curves is required.

From studies (unpublished data) of the nutritional requirements of lactic acid bacteria, it was observed that $d(-)$-glutamic acid had no activity for two strains (*Streptococcus faecalis* R (8043)[1] and *Leuconostoc citrovorum* (8082)), but was as active as $l(+)$-glutamic acid in promoting acid production by seven strains (listed in Table II). $d(-)$-Glutamic

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[1] The number given in parentheses after the name of the organism refers here, and elsewhere, in this paper to the number given by the American Type Culture Collection.

acid had variable, but only partial activity for eight strains of *Lactobacillus* (*i.e.*, *fermenti* 36 (9338), *gayonii* (8289), *pentosus* 124-2, *arabinosus* 17-5 (8014), *casei* (7469), *mannitopoeus, brassicae* (8041), and *brevis* (8257)), and for three strains of *Leuconostoc* (*i.e.*, *mesenteroides* (9135), *citrovorum* (8081), and *mesenteroides* P-60 (8042)). Assay procedures based on these findings were devised for the determination of $l(+)$-glutamic acid with

TABLE I

Composition of Basal Medium

Constituent	Mg. per liter	Constituent	Mg. per liter
dl-Alanine	667	Xanthine	12.0
Asparagine, natural	667	Dextrose	20,000
$l(+)$-Arginine·HCl	667	Sodium acetate	12,000
$l(-)$-Cysteine·HCl	667	Ammonium chloride	6,000
Glycine	667	KH_2PO_4	500
$l(-)$-Histidine·HCl·H_2O	667	K_2HPO_4	500
$l(-)$-Hydroxyproline	667	$MgSO_4$·$7H_2O$	200
dl-Isoleucine	667	$FeSO_4$·$7H_2O$	10
$l(-)$-Leucine	667	$MnSO_4$·$4H_2O$	10
dl-Lysine·HCl	667	NaCl	350
dl-Methionine	667	Thiamine·HCl	1.0
dl-Norleucine	667	Pyridoxine	1.6
dl-Norvaline	667	Pyridoxamine·2HCl	0.10
dl-Phenylalanine	667	Pyridoxal·HCl	0.10
$l(-)$-Proline	667	*dl*-Ca pantothenate	2.0
dl-Serine	667	Riboflavin	2.0
dl-Threonine	667	Nicotinic acid	2.0
dl-Tryptophane	667	Biotin	0.005
$l(-)$-Tyrosine	667	*p*-Aminobenzoic acid	0.10
dl-Valine	667	Folic acid*	0.005
Adenine sulfate·$2H_2O$	13.8	Choline chloride	10.0
Guanine·HCl·$2H_2O$	13.0	Inositol	25 0
Uracil	12.0		

* Product described previously (8).

Streptococcus faecalis R (8043) and of total $(d(-) + l(+))$-glutamic acid with any one of the seven organisms listed in Table II.

EXPERIMENTAL

The composition of the basal medium is given in Table I. Separate solutions of basal medium, amino acid test mixture, hydrolysates, standard amino acid, sodium chloride, and inocula were delivered into 3 inch test-tubes with the aid of an automatic pipette (Baltimore Biological Company instrument). The total volume of solution in each tube was 1.5 ml. All

of the solutions, except the basal medium, were adjusted to the same concentration of sodium chloride to compensate for any stimulatory or inhibitory salt effects. The standards ($d(-)$-, $l(+)$-, and dl-glutamic acid) were run in quadruplicate at eleven levels and the test mixture and the protein hydrolysates were run in duplicate at two to five levels. The time and temperature of incubation were 72 hours and 35°, respectively. The experimental results are given in Tables II to V.

DISCUSSION

It was found that $d(-)$- and $l(+)$-glutamic acid could be determined in an amino acid test mixture containing about a 3:1 ratio of $l(+)$- to $d(-)$-glutamic acid and in acid hydrolysates of casein and *Lactobacillus arabinosus* cells. As was shown in Table II, acid production by each of seven organisms was essentially the same for each standard ($d(-)$-, $l(+)$-, and dl-glutamic acid) at each of the eleven levels. Acid production by *Streptococcus faecalis* R was negligible for $d(-)$-glutamic acid and it was nearly equivalent for $l(+)$-glutamic acid and twice the quantity of dl-glutamic acid.

As was shown in Table III, the recovery of $l(+)$-glutamic acid from the amino acid test mixture was 100.4 per cent, while that of total ($d(-) +$ $l(+)$-glutamic) acid averaged 100.8 (95.0 to 105.2) per cent. The corrected $l(+)$-glutamic acid (Table IV) found for casein was 20.7 per cent and the total glutamic acid averaged 22.3 (21.4 to 23.3) per cent. Since the difference between these values is barely larger than the limits of accuracy of the microbiological procedures employed, the view that casein yields little, if any, $d(-)$-glutamic acid on acid hydrolysis appears to be correct. The $l(+)$-glutamic acid (Table V) found for dry, defatted *Lactobacillus arabinosus* cells was 4.23 per cent and the total glutamic acid averaged 8.23 (7.37 to 9.20 per cent). The calculated $d(-)$-glutamic acid was 4.00 per cent. The values reported previously (3) for $d(-)$- and $l(+)$-glutamic acid in the same cell preparation were 3.67 and 3.87 per cent, respectively.

Data for glutamic acid obtained by microbiological assay with *Lactobacillus arabinosus* 17-5 may be somewhat inaccurate, if the biological materials assayed contain both isomers of this amino acid, owing to the variable activity of $d(-)$- and $l(+)$-glutamic acid for this organism. It is of interest that *Lactobacillus arabinosus* 17-5 has been employed in all (published) microbiological assays for glutamic acid.

It is the object of future investigations to improve the precision and accuracy of the assay procedures and to determine the amounts and proportions of the antipodes of glutamic acid in a variety of natural materials.

TABLE II

Acid Production by Eight Lactic Acid Bacteria at Eleven Levels of d-, l-, and dl-Glutamic Acid*

Glutamic acid per tube	S. faecalis R (8043)			Leuconostoc dextranicum (8086)			L. mesenteroides (8293)			L. citrovorum (797)			L. citrovorum (7013)		
	l(+)-	dl-	d(-)-	l(+)-	dl-	d(-)-	l(+)-	dl-	d(-)-	l(+)-	dl-	d(-)-	l(+)-	dl-	d(-)-
							Glutamic acid† form								
γ															
0	1.68	1.69	1 73	2 25	2.26	2.18	2.28	2 28	2.29	2.71	2.79	2.80	2.89	2.89	2.89
2	1.81	1.70	1.72	2 95	2.82	2.87	3.50	3.46	3 50	3.95	3.88	3.91	4.10	3.98	3.93
4	2.38	1.80	1.75	3.86	3 60	3 64	4.68	4 80	4.85	4.90	5.19	5.24	5.14	5.23	5.20
6	2.83	2.14	1.75	4 52	4.35	4 40	5.62	5.71	5.80	6.04	6.20	6.20	5.99	6.12	6.15
8	3.32	2.40	1.73	5.20	5.05	5.11	6.37	6.52	6 65	6.67	6.82	7.06	6.61	6.93	7.02
10	3.73	2.60	1.70	5.98	5.82	5.89	7.01	7.21	7.20	7.35	7.52	7.60	7.30	7.55	7.55
15	4.95	3.20	1.75	7 06	7.13	7.15	8.14	8 35	8.39	8.40	8.60	8.70	8.36	8.66	8.71
17.5	5.44	3.54	1.77	7.77	7 77	7.76	8.63	8.88	8.95	8 87	9.25	9.31	8.84	9.11	9.19
20	5.92	3.73	1.72	8.13	8 25	8 35	9.05	9.28	9.36	9.28	9.60	9.77	9.08	9.37	9.54
22.5	6.22	4.01	1.75	8.45	8.74	8.84	9.39	9 68	9.69	9.65	9.95	10.25	9.44	9.72	9.92
25	6.53	4.28	1.72	8.70	9.12	9.31	9.77	10.09	10 18	10.16	10.30	10.50	9.72	9.84	10.07

Glutamic acid per tube	Lactobacillus lycopersici			L lycopersic§			L dextranicum (8359)			L. dextranicum (8358)		
					Glutamic acid† form							
	$l(+)$-	dl-	$d(-)$-	$l(+)$-	dl-	$d(-)$-	$l(+)$-	dl-	$d(-)$-	$l(+)$-	dl-	$d(-)$-
0	1.92	1.92	2.00	3.14	3.34	3.21	2.22	2.18	2.11	1.83	1.72	1.77
2	2.18	2.10	2.11	3.91	3.77	3 51	2.71	2.70	2.69	2.28	2.19	2.24
4	2.38	2.46	2.39	4 73	4 95	4 16	3.26	3.34	2 46	2 75	2 75	2.70
6	2.49	2.69	2.78	5 45	5 24	5 09	3 98	3.91	3.78	3 21	3.22	3.25
8	2.74	2.89	3.04	6.35	6.34	5 80	4.38	4.28	4.41	3.78	3.85	3.72
10	3.06	3.96	3.24	6 85	6 96	6.44	4 82	4.89	4.76	4.17	4.23	4.19
15	3.88	3 80	3.84	8 35	8.26	7.57	6 00	6.01	5.86	4 82	4.69	4.76
17.5	4.29	4.21	4.13	8 84	8.67	7.96	6.64	6.48	6.39	5 16	5.08	5.07
20	4.66	4.53	4.48	9 31	9.31	8.70	6.88	6.82	6.84	5.22	5.15	5.15
22.5	5.02	4 86	4.71	9 91	9.85	9.11	7.12	7.33	6 95	5.22	5.22	5.16
25	5.21	5.27	5.12	10.60	10.69	9.85	7.59	7.53	7 36	5.38	5.15	4 98

* The acid production is given as ml. of 0 01 N base to titrate 1 ml. of final solution. The numbers given in parentheses after the names of the organisms in the tables and the text are those given by the American Type Culture Collection

† The $d(-)$-glutamic acid employed in the present experiments was obtained through the courtesy of Dr. H. S. Olcott, Western Regional Research Laboratory, Albany, California. The $d(-)$-glutamic acid was isolated from the polyglutamic acid produced by Bacillus subtilis. This sample was considered to be nearly analytically pure, since its specific rotation, $[\alpha]^{24}_D = -31.73°$ in 1.73 N HCl with $c = 7$, was nearly identical with that, $+31.71°$, found for a sample of highly purified $l(+)$-glutamic acid under the same conditions. These measurements were made by Mr. R. C. Bovie in the writers' laboratory.

‡ Basal medium given in Table I.

§ Basal medium given in Table I, except that arabinose (1 per cent) and xylose (1 per cent) were substituted for glucose (2 per cent).

TABLE III

*Per Cent Recovery of l(+)- and Total Glutamic Acid from Amino Acid Test Mixture**

Amino acid test mixture, level per tube	l(+)-Glutamic acid	Total glutamic acid							
	S fae-calis R (8043)	Leuco-nostoc dextran-scum (8086)	L. mesen-teroides (8293)	L. cit-rovorum (797)	L cit-rovorum (7013)	Lacto-bacillus lycoper-sici† (4005)	L lyco-persici‡ (4005)	L dex-traniscum (8359)	L dex-traniscum (8358)
γ									
470	105.2	100.0	92.5	87.5	98.8	102.2	77.5	113.7	77.5
940	101.3	100 6	106.9	110.2	107.2	107.2	104.9	96.8	75.0
1410	100.0					100.0	102.2		
1880	95.3								
Average	100.4	100.3	99 7	98.8	103 0	103.1	94.9	105.2	76.2§

* The amino acid mixture contained 1 27 per cent $l(+)$-glutamic acid and 0 424 per cent $d(-)$-glutamic acid; otherwise, it was the same as amino acid test mixture No. 4 described previously (9).

† Basal medium given in Table I.

‡ Basal medium given in Table I, except that arabinose (1 per cent) and xylose (1 per cent) were substituted for glucose (2 per cent).

§ Omitted in calculating the average value referred to in the text.

TABLE IV

Per Cent of l(+)- and Total Glutamic Acid in Casein

Casein* level per tube	l(+)-Glutamic acid	Total glutamic acid							
	S fae-calis R (8043)	Leuco-nostoc dextran-scum (8086)	L mesen-teroides (8293)	L cit-rovorum (797)	L. cit-rovorum (7013)	Lacto-bacillus lycoper-sici† (4005)	L lyco-persici‡ (4005)	L dex-traniscum (8359)	L dex-traniscum (8358)
γ									
18.6	20.8	21.5	20.6	23.6	22.1	29.5§	24.7	22.1	19.8
37 3	20.5	21.2	21.4	21.5	21.5	21.7	23.5	21.2	21.2
56 0	21.3	21.0	22.3	23.6	21.9	23.3	22.2	23.8	21.7
74.6	20.6	22.4	23 2	22.8	22.8	23.5	24.2	20.2	22.6
93 2	20.2	24.2	23.4	23.6	25 0§	24.0	21.9	19.6	17.8§
Average	20.7	22.1	22.4	23.0	22 1	23.1	23.3	21.4	21.3

* Corrected for moisture and ash.

†, ‡, § See the foot-notes to Table III.

*Per Cent of l(+)- and Total Glutamic Acid in Lactobacillus arabinosus Cells**

L arabinosus cells, levels per tube	l(+)-Glutamic acid	Total glutamic acid							
	S faecalis R (8043)	Leuconostoc dextranscum (8086)	L mesenteroides (8293)	L citrovorum (797)	L citrovorum (7013)	Lactobacillus lycopersicit (4005)	L. lycopersicit (4005)	L dextranicum (8359)	L dextranicum (8358)
γ	.								
60	3 97	8 12	7 50	7.80	7 80	10 60	7.70	8 73	7.57
120	4.02	8.07	8.34	8.83	8 20	9.95	7.84	8 00	7.84
180	4 43	8 16	8.70	9.04	8 96	8.60	7.70	8.00	7.24˙
240	4 25	8.20	8 98	9.17	9.94	8.56	8 00	6.73	6.83
300	4.50					8.27	7.50	6.83	4.40§
Average	4.23	8 14	8 38	8.71	8.72	9.20	7.75	7.66	7.37

* The cells were those referred to under the symbol, O_2, given in a foot-note of a previous paper ((3) p. 75).

†, ‡, § See the foot-notes to Table III.

SUMMARY

It has been found that $d(-)$-glutamic acid has no activity for *Streptococcus faecalis* R (8043) and *Leuconostoc citrovorum* (8082), and that it has activity equivalent to $l(+)$-glutamic acid for seven organisms. $d(-)$-Glutamic acid has variable, partial activity for eleven organisms.   It has been shown that $d(-)$- and $l(+)$-glutamic acid can be determined with reasonable accuracy in an amino acid test mixture containing about a 3:1 ratio of $l(+)$- to $d(-)$-glutamic acid.   It is concluded from the assay data that casein yields little, if any, $d(-)$-glutamic acid, and that *Lactobacillus arabinosus* cells yield about 4 per cent each of $l(+)$- and $d(-)$-glutamic acid.

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THE DETERMINATION OF GLYCINE IN PROTEIN HYDROLYSATES WITH LEUCONOSTOC MESENTEROIDES P-60[*]

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The determination of glycine in protein hydrolysates with *Leuconostoc mesenteroides* P-60 is described in this communication. Although glycine has been reported to be essential, or accessory, to the growth of *Streptococcus faecalis* (*S. lactis* R) (2), *Lactobacillus brevis* (3), *Lactobacillus pentoaceticus* (3), *Leuconostoc citrovorum* (3), and *Leuconostoc mesenteroides* P-60 (3, 4), it has been determined microbiologically only with the last organism[1] (5).

EXPERIMENTAL

The assay techniques employed were essentially the same as those described previously (6). The protein hydrolysates, the inoculum suspensions and solutions of the basal media, amino acid test mixtures, the standard amino acid, and sodium chloride were delivered to 4 inch test-tubes with the aid of an automatic pipette (Baltimore Biological Company). The final volume was 2 ml. per tube. The solutions of all of the materials, except the basal media, were adjusted to the same concentration of sodium chloride to compensate for any stimulatory or inhibitory effects. The standard was run at fifteen levels (0 to 28 γ), the amino acid test mixtures and the protein hydrolysates were run at five levels, and five to six replicate tubes were employed at each level of sample and standard. The composition of the basal medium was the same as that given as Medium D, Table I, in Paper XVIII (4), except that glycine was omitted and the concentration of total amino acids was twice that stated in Table I, Paper XVIII. Glycine was added to the basal medium to overcome an induction period which extended from 0 to about 7 γ of glycine. Usually 3 γ of glycine per ml. of final solution per tube were added. It was found from

[1] In β-lactoglobulin by an unpublished method.

experiments with multiple levels of total amino acids (1.3, 2.0, 2.7, and 3.3 times the quantities given in Medium D, Table I, Paper XVIII) and varying concentrations of glycine (10, 20, 30, and 40 γ per 2 ml. of final

TABLE I

Assay of Glycine in Amino Acid Test Mixture 1* (Glycine Content 1.49 Per Cent)

Amino acid mixture per tube	Glycine present per tube	Titration volume†	Glycine found per tube	Glycine recovered‡
γ	γ	*ml.*	γ	*per cent*
284.8	4.23	3.50	3.92	92.7
569 6	8 46	4.97	7.95	94 0
854.4	12 70	6.32	12.70	100 0
1139	16 93	7.52	17.40	102 8
1424	21.16	8 08	20.80	98.3
Average.				97.5

* The composition of the test mixture, except for the increased glycine content, is shown in Table IV of Paper XVII (7).

† The contents of six replicate tubes at each level of sample were mixed and titrated with 0.124 N NaOH. The preferred conditions (see the "Discussion") were employed.

‡ The recoveries in analogous experiments from a test mixture containing 0 866 per cent glycine were 103 and 104 per cent and from one containing 0.292 per cent glycine were 112 and 108 per cent.

TABLE II

Assay of Glycine in Amino Acid Test Mixture 2* (Glycine Content 1 075 Per Cent)

Amino acid mixture per tube	Glycine present per tube	Titration volume†	Glycine found per tube	Glycine recovered
γ	γ	*ml*	γ	*per cent*
372	4.00	3.43	3 70	92.5
744	8.00	4.90	7.75	96.9
1116	12.00	6.28	12.60	105.0
1488	16.00	7.40	16 80	105.0
1860	20.00	8 20	21.55	107.8
Average. . .				101.4

* The composition of the test mixture is that given in Table II of Paper XVIII (4).

† The contents of six replicate tubes at each level of sample were mixed and titrated with 0.124 N NaOH. The preferred conditions (see the "Discussion") were employed.

volume per tube) that acid production was nearly constant under these conditions.

The experimental results are given in Tables I to IX.

DISCUSSION

It has been found that glycine could be determined most accurately in samples containing widely varying concentrations of this amino acid under the following conditions: an inoculum of *Leuconostoc mesenteroides* P-60

TABLE III

Assay of Glycine in Amino Acid Test Mixture 3* (Glycine Content 27.96 Per Cent)

Amino acid mixture per tube	Glycine present per tube	Titration volume†	Glycine found per tube	Glycine recovered
γ	γ	ml	γ	*per cent*
18.8	5.26	2.88	4.85	92.2
37.6	10.51	4.54	10.52	100.1
56.4	15.77	5.65	15 70	99.5
75.2	21.02	6.65	21.10	100.4
94.0	26 28	7.55	26 84	102.1
Average.. . . .			.	98.9

* The composition of the test mixture is given in Table III of Paper XIX (8).

† The six replicate tubes were titrated separately with 0.027 N NaOH. The average of each set of six titration values is given in Table III. The basal medium was Medium D given in Table I of Paper XVIII (4), except that the level of total amino acids was 1 33 times that stated   The incubation time was 2 days.

TABLE IV

Assay of Glycine in Casein Hydrolysate*

Hydrolyzed casein per tube (moisture- and ash-free)	Titration volume†	Glycine found per tube	Glycine in casein
γ	ml	γ	*per cent*
223.7	3.45	4.16	1.86
447.4	5 00	8.68	1 94
671.0	6.38	13.30	1.98
894.7	7 51	18.00	2.01
1118	8.40	22.00	1.97
Average .		.	1.95

* The preferred conditions (see the "Discussion") were employed.

† The contents of six replicate tubes at each level of sample were mixed and titrated with 0.124 N NaOH.

diluted with a volume of sterile saline from 3 to 9 times that of the medium from which it was centrifuged, a basal medium containing 3 γ of glycine per ml. of final solution, and total amino acids at twice the level employed previously for the determination of lysine (8) and histidine (10), and in-

cubation for 3 days at 35°.   Under these conditions, the average mean deviation from the mean values (five levels of sample) averaged about 2 per cent, the recoveries of glycine from amino acid test mixtures and pro-

TABLE V

Recovery of Glycine Added to Casein Hydrolysate*

Hydrolyzed casein per tube (moisture- and ash-free)	Glycine			
	In casein per tube†	Added per tube	Found per tube	Recovered‡
γ	γ	γ	γ	*per cent*
111.8	2.18	2.00	4.28	105.0
223.7	4 35	4.00	8.32	99.2
335.5	6.54	6.00	12.75	103.5
447.4	8.71	8.00	15 20	81.1
559.2	10.89	10.00	21.42	105.3
Average.	. .	.	.	98.8

* The sample was prepared to contain 10.0 γ of glycine and 559.2 γ of moisture- and ash-free casein per ml.   Volumes from 0.20 to 1.00 ml. were taken for the assays. The preferred conditions (see the "Discussion") were employed.

† Estimated on the basis of the 1.95 per cent of glycine in casein given in Table IV.

‡ Calculated by the method given in Paper XXV (9).

TABLE VI

Assay of Glycine in Silk Fibroin Hydrolysate*

Hydrolyzed silk fibroin per tube (moisture- and ash-free)	Titration volume†	Glycine found per tube	Glycine in silk fibroin
γ	*ml.*	γ	*per cent*
11.64	3.82	5.20	44.6
23.28	5.53	10.40	44.6
34.92	7.08	15.95	45.7
46.56	8.30	21.42	46.0
58.21	9.05	25.50	43.8
Average	. .	. . . . .	44.9

* The preferred conditions (see the "Discussion") were employed.

† The contents of six replicate tubes at each level of sample were mixed and ti- trated with 0 120 N NaOH.

tein hydrolysates were approximately 100 per cent, and the assay values for glycine in casein and for glycine in silk fibroin agreed closely.   Rep- resentative assays under these conditions are given for glycine in three amino acid test mixtures (Tables I to III), glycine in hydrolyzed casein

TABLE VII

*Recovery of Glycine Added to Silk Fibroin Hydrolysate**

Hydrolyzed silk fibroin per tube (moisture- and ash-free)	In silk fibroin per tube†	Added per tube	Found per tube	Recovered‡
γ	γ	γ	γ	*per cent*
5.82	2.61	2.00	4.60	99.5
11.64	5.23	4.00	9.24	100.2
17.46	7.84	6.00	14.06	103.7
23.28	10.45	8.00	18.28	97.9
29.10	13.06	10.00	22.16	91.0
Average		.	. ...	98.5

* The sample was prepared to contain 10.0 γ of glycine and 29.10 γ of moisture- and ash-free silk fibroin per ml. Volumes from 0.20 to 1.00 ml. were taken for the assays. The preferred conditions (see the "Discussion") were employed.

† Estimated on the basis of the 44.9 per cent of glycine in silk fibroin given in Table VI.

‡ Calculated by the method given in Paper XXV (9).

TABLE VIII

*Summary of Recovery Data for Glycine from Amino Acid Test Mixtures**

		Amino acid Test Mixture No.		
		1	2	3
		per cent	*per cent*	*per cent*
Preferred conditions†	A.D ‡	3.6	5.0	3.9
	Range§	97.5–101.7	99.9–103.2	104.6
	Mean‖	100 2 ± 1.8 (3)	102.0 ± 1.1 (5)	104.6 ± 3.9 (1)
All other conditions¶	A.D.	4.4	2.8	1.7
	Range	90–128	87–112	98.6–104.2
	Mean	113 ± 9 (25)	102.4 ± 3.2 (23)	100.8 ± 1.8 (9)

* See Tables I to III for the composition of test mixtures.

† See the "Discussion "

‡ Average mean deviation from the mean of the values at the five levels of the sample.

§ Range of all assay values.

‖ Mean assay value ± its mean deviation from the mean. The figures in the parentheses denote the number of assays.

¶ The concentrations of amino acids varied from 1.33 to 3.33 times that employed under the preferred conditions; the incubation times varied from 2 to 6 days and the dilution of the inoculum suspension varied from 1:3 to 1:81.

with and without added glycine (Tables IV and V), and glycine in hydro-lyzed silk fibroin with and without added glycine (Tables VI and VII). The results of the assays of glycine in the three amino acid test mixtures

under all of the experimental conditions employed are summarized in Table VIII and the results of the assays of casein and silk fibroin for glycine ·are given in Table IX.

It appears significant, however, that the accuracy with which glycine could be recovered from amino acid test mixtures decreased markedly as the glycine decreased from 1.486 to 0.292 per cent (see Table I). Analogous results were obtained previously in the assay of phenylalanine (6). That the accuracy with which glycine (or other amino acids) may be determined microbiologically in a protein hydrolysate is a function of its concentration is further indicated by the observation that the recovery of glycine under all of the experimental conditions approached the theoretical amount only from the amino acid test mixture (No. 3) in which glycine was present in

TABLE IX

Summary of Protein Assay and Recovery Data

		Glycine in casein*		Glycine in silk fibroin†	
		Found	Recovered	Found	Recovered
	*	*per cent*	*per cent*	*per cent*	*per cent*
Preferred conditions	A D.	3.7	3 6	3 0	2 9
	Range	1.82–1 95	98.4–102 6	42.8–45 0	94 7–105.6
	Mean	1.90	100.1	43 6	99 5
		± 0 04 (3)	± 1.6 (3)	± 1.0 (3)	± 4 1 (3)
All other conditions	A.D.	3.9	3 0	1.5	3 0
	Range	1.58–2 12	91–108	41 9–44 5	95 1–105.7
	Mean	1.93	102 4	43 4	101 0
		± 0 11 (23)	± 3.1 (23)	± 0.9 (8)	± 3.2 (8)

* Corrected for the 6.21 per cent moisture and the 0.55 per cent ash in the prepara- tion described in Paper XVII (7).

† Corrected for the 5.68 per cent moisture and the 0.25 per cent ash in the prepara- tion described in Paper XVII (7).

relatively high concentration (28 per cent). The value of amino acid test mixture experiments in assessing the dependability of an analytical procedure, first established by Osborne and Jones (11) in 1910, has been emphasized recently by Martin and Synge (12).

It is of interest that an induction period extending up to about 7 γ of glycine for *Leuconostoc mesenteroides* P-60 under the stated experimental conditions could be overcome satisfactorily for assay purposes by the addition of 3 γ of glycine per ml. of final solution. The same phenomenon has been observed for *Lactobacillus arabinosus* 17-5 and some other organ- isms at low levels of glutamic acid (13–15), and Lyman *et al.* (14) utilized a basal medium for the assay of glutamic acid to which was added the

equivalent of 0.25 mg. of glutamine per tube. It has been proposed that glutamine rather than glutamic acid is the active metabolite for *Lactobacillus arabinosus* (14, 15) and some other organisms, but it does not seem probable that induction occurred in the present experiments because of the slow synthesis of some unknown substance possessing higher activity for *Leuconostoc mesenteroides* than glycine. A plausible explanation would appear to be that another structurally similar amino acid such as alanine exerted a depressive action at low levels of glycine. Lewis and Olcott (13) have found that aspartic acid was more effective than any other amino acid tested in depressing acid production by *Lactobacillus arabinosus* on a basal medium containing glutamic acid-free casein hydrolysate. These authors found, also, that glutamic acid activity was depressed significantly by arginine. The induction periods which have been noted in this laboratory for valine (*Leuconostoc mesenteroides*), phenylalanine (*Lactobacillus fermenti*), and serine (*Lactobacillus casei*) may be due to specific antagonism by leucine, tyrosine, and threonine, respectively.

Glycine in Casein

It has been found by assay with *Leuconostoc mesenteroides* P-60 under the preferred conditions that casein, corrected for moisture and ash, contained 1.90 (1.82 to 1.95, range of three assays) per cent of glycine. The average value, 1.93 (1.58 to 2.12, range of twenty-three assays) per cent, was obtained under all other conditions.

Martin and Synge (12) have stated that "the least satisfactory aspects of quantitative amino acid analysis seem at present to be (*i*) the determination of glycine and hydroxyproline; (*ii*) the differentiation of leucine and isoleucine." Only traces of glycine have been found in casein (16–19) by Fischer's ester method. Although Fischer (18) recovered 78 per cent of the 5 gm. of glycine added to 25 gm. of hydrolyzed casein, it seems unlikely that as high a percentage of the relatively small amount of glycine present normally in casein could be isolated by the ester procedure. 40 to 80 per cent of eleven amino acids in a test mixture was recovered by Osborne and Jones (11) in a study of the ester method but glycine was not investigated.

Values ranging from 0.4 to 1.0 per cent of glycine in casein have been reported by workers (20–23) who employed an *o*-phthalic dialdehyde colorimetric procedure (21, 24, 25). Since histidine (26), as well as tryptophane, cystine, arginine, alanine, asparagine, and ammonium salts (25), gives colored products with this reagent, it seems unlikely that quantitative results would be readily obtainable by this method. It may be significant that the 22.2 per cent of glycine obtained (21) for gelatin by this colorimetric procedure was considerably lower than the values (25.5 to 27 per

cent) found by the solubility product (26) and the trioxalatochromiate
(27) methods. It is also of interest that no glycine could be detected in
gramicidin (28) by the Patton colorimetric procedure, while two residues
of glycine per thirty residues of total amino acids were found by means of
the flowing chromatogram (29).

About 0.5 per cent of glycine has been isolated from casein as the picrate
(30), 0.3 per cent (31) as the nitranilate, and 3.6 per cent (32) as the ni-
tranilate. The last value may be high, since the purity of the glycine
nitranilate isolated from casein was not established. The product may
have been contaminated with the nitranilates of some other amino acids
since Stein, quoted by Block (33), has stated that high values were obtained
by Town's method probably because of the partial precipitation of the
basic amino acids along with glycine. It is of interest that a method for
the determination of histidine as the nitranilate has been described by
Block (33, 34).

Glycine in Silk Fibroin

It has been found that silk fibroin, corrected for moisture and ash, con-
tained 43.6 (42.8 to 45.0 range of three assays) per cent determined under
the preferred conditions and 43.4 (41.9 to 44.5, range of eight assays)
determined under all other conditions. It seems probable that the average
value, 43.6 per cent, is approximately correct, since it is in close agreement
with the average value, 43.8 (43.3 to 44.4, range of three determinations)
per cent, determined by the 5-nitronaphthalene-1-sulfonate (35) method
and the average value, 43.8 (43.5 to 44.1, range of three determinations)
per cent, determined by the trioxalatochromiate procedure (36). Boyd
(37) reported that silk fibroin contained 42 per cent of glycine, determined
gravimetrically as its hydantoin, while values ranging from 9.8 to 40.5 per
cent have been found (38–57) by Fischer's ester method.

SUMMARY

A microbiological method has been described for the determination of
glycine in protein hydrolysates with *Leuconostoc mesenteroides* P-60. The
preferred assay conditions were considered to be 3 days incubation at 35°
and a basal medium of the composition reported previously (4), except that
the concentration of total amino acids was twice that given and 3 γ of gly-
cine were added per ml. of final volume.

It was concluded that the probable true value for glycine in casein is
1.9 ± 0.1 per cent and in silk fibroin 43.6 ± 1.0 per cent. The value for
casein is somewhat higher than any other reported in the literature (ex-
cept that found by Town (32)), but that for silk fibroin is in close agree-

ment with the most reliable values which have been obtained by other workers.

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KIDNEY PHOSPHATASE IN ALIMENTARY HYPERGLYCEMIA AND PHLORHIZIN GLYCOSURIA. A DYNAMIC MECHANISM FOR RENAL THRESHOLD FOR GLUCOSE

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PLATE 1

Glucose, which appears in the glomerular filtrate at all levels of blood sugar, is reabsorbed mainly in the proximal convoluted tubules of the kidney. This has been established by Walker and Hudson (1) by means of micro dissection and microanalytical techniques. Under usual physiological conditions, the reabsorption of the sugar is complete. The renal threshold for glucose may hence be defined as that level of blood glucose beyond which complete tubular reabsorption no longer occurs. Involvement of the phosphorylation process in the mechanism of reabsorption of glucose was postulated by Lundsgaard and his colleagues (2, 3) in the explanation of phlorhizin glycosuria. Beck (4) studied the effect of phlorhizin on phosphorylation and dephosphorylation, and partially confirmed Lundsgaard's views, although Beck's positive findings with reference to dephosphorylation were limited to acid phosphatase. It is of interest that histochemical staining techniques (5, 6) suggest that the proximal tubules are one of the preferential sites of high concentration of the kidney phosphatase. Recent reviewers (7, 8), on the other hand, have regarded the evidence for the rôle of phosphatase in tubular glucose reabsorption and particularly the inhibition of kidney phosphatase by phlorhizin as entirely inadequate. It should be pointed out that the work of Lundsgaard and his colleagues (2, 3) and Beck (4) has been confined to experiments *in vitro*. As far as we know, a demonstration of the relation of phosphorylation-dephosphorylation in glucose reabsorption in kidney tubules has not been demonstrated *in vivo*.[1]

One of the difficulties in earlier work is the confusion which has resulted from a lack of distinction between phosphorylation and dephosphorylation. It is now recognized that, under the conditions existing in tissues, the position of equilibrium is such that the catalysis by phosphatase is essentially non-reversible. Phosphatase catalyzes only the dephosphoryla-

[1] Personal communication to one of us (D. L D.) from E. Lundsgaard.

tion of certain phosphate esters. The phosphorylation process is biologically under the control of separate, complex enzyme systems involving hexokinase and adenosine triphosphate (ATP). It should be understood, therefore, that studies of phosphatase are directed mainly towards the dephosphorylation reaction. However, as will be seen later, in biological systems the separation of the dephosphorylation and phosphorylation process may be difficult. In the present investigation of the possible rôle of phosphorylation-dephosphorylation in the renal threshold for glucose, attention has been directed to a study of phosphatase activity in two conditions: induced alimentary hyperglycemia with glycosuria and phlorhizin glycosuria. The study of the hyperglycemic state was undertaken, since it may be regarded as more physiological than phlorhizin poisoning. It was postulated that in acute hyperglycemia the tubular cells would be working at a maximum to reabsorb glucose, and, therefore, involvement of the phosphatase mechanism might be susceptible of demonstration. An improved method for the quantitative preparation of phosphatase extracts of kidney has been developed. It has been discovered from such extracts that hyperglycemia induces a significant increase in the level of both acid and alkaline phosphatase activity. This unexpected and interesting phenomenon received confirmatory evidence for its existence by a semiquantitative application of Gomori's histochemical staining technique (5). Moreover, it has been clearly demonstrated that the addition of phlorhizin inhibits markedly both the acid and alkaline phosphatase activity of the kidney extracts, and that the phosphatase activity of phlorhizinized rat kidneys is diminished. As an aid in interpretation, data have also been gathered on certain aspects of the phosphorylation process in kidney.

Methods

Extraction of Phosphatase—Albino rats of both sexes, 200 to 500 gm. in weight, fasted for 24 hours, were used. The kidneys were secured by unilateral or bilateral nephrectomy, rapidly performed under moderate ether anesthesia. After as much connective tissue as possible was quickly removed, and the excess blood blotted with filter paper, the kidney was frozen with liquid air and ground to a powder in a mortar. This is essentially the procedure of Perlmann and Ferry (9). In preliminary studies of extraction procedures we have found that homogenization of the above powder results in higher phosphatase activity (appreciably higher than that reported for kidney by Beck (4)). An additional step was accordingly introduced: 0.2 to 0.3 gm. of the powder, representing approximately one-fifth of the original rat kidney, was transferred to a weighed test-tube homogenizer of the Potter-Elvehjem type (10). The tube was reweighed,

10 ml. of 0.5 M NaCl added, and the material homogenized for 2 minutes at room temperature. In the experiments involving phlorhizin greater concentration was required, and hence approximately 0.5 gm. of kidney powder was homogenized with 1.5 ml. of 0.5 M NaCl.

Determination of Phosphatase Activity—Except in the phlorhizin experiments, 1 ml. of the homogenate was added to 9 ml. of a buffered sodium β-glycerophosphate solution, prepared according to Shinowara, Jones, and Reinhart (11), and previously adjusted to 37.5°. In the experiments with phlorhizin, acid phosphatase activity was measured with 0.5 ml. of the concentrated homogenate and 1.0 ml. of the buffered substrate (Shinowara *et al.*), pH 4.9 to 5.1, while the determination of alkaline phosphatase required special conditions. 0.5 ml. of the concentrated homogenate was added to 1.0 ml. of a solution containing 5.0 gm. of sodium β-glycerophosphate and 4.24 gm. of sodium diethyl barbiturate per 100 ml. The mixture was then adjusted to pH 9.2 by addition of 0.5 N acetic acid.

The mixture of kidney extract and buffered substrate was incubated in a water thermostat for 1 hour at 37.5°. 5 ml. of 10 per cent trichloroacetic acid were then added to inactivate the enzyme. In each determination of phosphatase activity, aliquots, in which the enzyme was inactivated by addition of trichloroacetic acid at the start of incubation, were used as controls to correct for the inorganic phosphate originally present in extract and substrate. Both the experimental and control solutions were filtered after inactivation. The inorganic phosphate present in an aliquot (usually 2 ml.) of each filtrate was determined by the method of Fiske and Subbarow (12), with a Klett-Summerson (13) photoelectric photometer with the red (λ 660 mμ) filter. The pH of the buffered sodium β-glycerophosphate solutions was checked by a glass electrode. Blood sugar was determined by the micromethod of Folin and Malmros (14), also by means of the photoelectric photometer but with the green (λ 540 mμ) filter.

Determination of Adenosine Triphosphate—0.5 to 1.0 gm. of powdered frozen kidney tissue was added to 7 ml. of 7 per cent trichloroacetic acid in a tared centrifuge tube at 0°, according to the procedure of Kaplan and Greenberg (15). The 7 minute-hydrolyzable phosphate was determined directly on the trichloroacetic acid filtrate made up to a volume of 25 ml.

Determination of Aerobic Phosphorylation—The method used was essentially that of Colowick, Welch, and Cori (16). Both oxygen consumption by Warburg's direct manometric technique (17) and decrease in inorganic phosphate were measured. The kidneys were removed from decapitated rats and immediately placed on cracked ice, weighed, and transferred to a homogenizer tube kept in ice. Sufficient 0.05 M phosphate buffer of pH 7.5 was added to make the concentration approximately 180 mg. of tissue per 1 ml. of homogenate. The material was homogenized for 1 minute.

1 ml. aliquots of this homogenate were then added to the Warburg vessels containing 1 ml. of substrate and 0.2 ml. of 2 N NaOH in the center well. The composition of the substrate per ml. was glucose 10 mg., NaF 3 mg., magnesium citrate 0.2 mg., and $l(+)$-glutamic acid 4 mg., adjusted to pH 8.0 with 1 N NaOH. The vessels were gassed with 100 per cent oxygen for 2 minutes, and equilibrated at 38.3° for 8 minutes. Oxygen consumption was then measured for the next 10 minutes. After 30 minutes, the vessels were disconnected from the manometers and 2 ml. of 7 per cent trichloroacetic acid were added. The contents were transferred to 100 ml. volumetric flasks, brought to volume, and filtered. The inorganic phosphate was determined on aliquots of the filtrate. At the start of the incubation period trichloroacetic acid was added to one of the vessels, the filtrate of which was used for the measurement of the inorganic phosphate originally present in homogenate and substrate.

<div align="center">EXPERIMENTAL</div>

The data collected in Tables I and II indicate that alimentary hyperglycemia was accompanied by a statistically significant (18) increase in the acid and alkaline phosphatase activities of rat kidney homogenates. The increase amounted to 55 per cent for the acid and 70 per cent for the alkaline phosphatase. That this rise was not accounted for by dehydration of the kidney tissue as a consequence of the administration of hypertonic glucose to a fasted animal was evident from the constancy of the wet weight-dry weight ratios.

As an independent check on these results, kidney sections from a fasted and from a hyperglycemic rat were stained for acid phosphatase by the Gomori technique (5). By limiting the incubation period to only 7 hours at 37.5°, it was possible to make a semiquantitative demonstration by this method. In Fig. 1, A, the tissue from the fasted rat showed only very slight staining for "phosphatase activity," whereas in Fig. 1, B, the kidney section from the hyperglycemic animal was stained very heavily.

In Table III we present data showing that phlorhizin added in vitro was capable of inhibiting markedly both acid and alkaline phosphatase activity of rat kidney extracts. The magnitude of the inhibition depended on the phlorhizin concentration. In contrast with the findings of Beck (4), in our experiments the effect was more pronounced in the pH range of alkaline phosphatase.

Evidence that the inhibitory effect of phlorhizin on kidney phosphatase activity can also be demonstrated in vivo is furnished in Table IV. We could not demonstrate the effect of the drug when we measured the activity of the dilute kidney homogenates from phlorhizinized rats. In the successful demonstration, the concentrated homogenates previously described,

TABLE I

Acid Phosphatase Activity, at pH 4.9 to 5.1, per Gm., Wet Weight, of Kidney Tissue in 24 Hour-Fasted and Hyperglycemic Rats

Rat No.	State	Glucose dosage*	Time of analysis†	Blood sugar	Ratio, wet to dry weight	Phosphatase activity‡
		gm.	hrs	mg. per 100 ml.		mg. P per hr.
1	Fasted					4.4
2	"					6.1
3	"					4.2
4	"				3.98	4.2
5	"					4.6
6	"					6.4
7	"					5.5
8	"			95		3.5
16§	"					4.6
17§	"					6.5
18§	"					4.5
19§	"					3.5
20	"					6.8
21	"					6.1
22	"					6.8
23	"					9.1
24	"					7.1
25	"					3.6
27	"			79		3 8
28	"			71		4.1
29	"			130	4 36	5.9
30	"			95	3.62	5.3
35	"				4.60	
37	"				4.73	
9	Hyperglycemic	3 0	1 00			8.0
10	"	3.0	2.50			7.3
11	"	3.0	2.25			6.3
12	"	3.0	1.75			9.4
13	"	3.0	2.25			7.0
14	"	3 0	4 00			7.3
15	"	3 0	2 00	160		11.6
16§	"	3.0	2 00			5.4
17§	"	3.0	2 00		3 21	7.3
18§	"	3.0	2.00			6.7
19§	"	3.0	2.00			6.3
26	"	1.0	1.00			11.2
31	"	1.0	1.00	155		7.1
32	"	1.3	1.50	197		7.6
33	"	1.0	1.50	236		8.8
34	"	1.0	3.50			11.9
46	"	1.0	1.50	223	3.97	10.4
47	"	3.0	2.00		4.18	

TABLE I—*Concluded*

Rat No.	State	Glucose dosage*	Time of analysis†	Blood sugar	Ratio, wet to dry weight	Phosphatase activity‡
		gm	*hrs.*	*mg. per 100 ml.*		*mg. P per hr.*
48	Hyperglycemic	1.0	1.00		4.29	
49	"	1.0	1.00		4.57	
61	"	1.0	2.00		4.38	
62	"	1.0	2.00		4.35	
63	"	1.0	1.75		4.87	
64	"	1.0	2.00		4 27	
Fasted, mean ± s.e ‖					4 26 ± 0 20	5 30 ± 0.31
Hyperglycemic, mean ± s.e.‖					4 23 ± 0 16	8.21 ± 0.48

* Total amount of glucose, administered by stomach tube to fasted animals in a. 50 per cent solution, 2 ml. per hour, usually for 3 hours.

† Interval of time between the last dose of glucose and nephrectomy

‡ Expressed as mg. of P liberated in 1 hour from sodium β-glycerophosphate at 37.5°. This is equivalent to Bodansky units (19).

§ In Rats 16 to 19 unilateral nephrectomy was performed to obtain the values in the fasting state, while the analyses after hyperglycemia were carried out 2 weeks later on the remaining hypertrophied kidney.

‖ Standard error = $\pm\sqrt{\Sigma d^2/n(n-1)}$. The value of t (Fisher (18)) for the difference between the means of fasted and hyperglycemic rats is 5 27 with a probability, P, of less than 0 01 that this difference may be attributable to chance

in which the concentration of phlorhizin in the final extract was about one-fourth its concentration in the original kidney tissue, were studied. While values for phosphatase activity on such concentrated homogenates were relatively low (compare the values in Table IV with those in Tables I to III), nevertheless they were reproducible and justified comparison of values before and after phlorhizin. Both acid and alkaline phosphatase activities were definitely lower in the phlorhizinized animals than in the normal controls. Experiments were performed which suggest that the inability to detect the action of phlorhizin in the dilute homogenates may be attributable to the dilution of the drug (and this factor may explain some of the discordant results in the literature). When aliquots of the concentrated homogenates from the phlorhizinized animals were diluted 10-fold, they then yielded essentially normal values for phosphatase activity (for example, in Rat 40, Table IV, upon dilution of the homogenate the activities of the acid and alkaline phosphatase became, respectively, 5.6 and 45 mg. of P per hour). Furthermore, the inhibitory effect of phlorhizin on phosphatase could be removed by dialyzing homogenates to which the drug had been added against 0.5 M NaCl. (In one experiment, the activi-

ties before and after dialysis were, respectively, 5.3 and 6.3 mg. of P per hour for the acid and 17.6 and 20.5 mg. of. P per hour for the alkaline phosphatase.) The increase in kidney phosphatase activity, observed in normal animals rendered hyperglycemic, was also found in phlorhizinized

TABLE II

Alkaline Phosphatase Activity, at pH 9.2 and 8.3, per Gm., Wet Weight, of Kidney Tissue in 24 Hour-Fasted and Hyperglycemic Rats

Rat No.	State	Glucose dosage*	Time of analysis*	pH	Phosphatase activity*
		gm.	*hrs.*		*mg. P per hr.*
20	Fasted			9.2	51
21	"		·	9.2	37
22	"			9.2	46
23	"			9.2	54
24	"			9.2	55
25	"			9.2	46
26	Hyperglycemic	1.0	1.00	9 2	100
34	"	1.0	3.50	9 2	76
106	"	1.0	1 00	9.2	87
107	"	1 0	1.00	9 2	77
94	Fasted			8 3	21
95	"			8 3	18
96	"			8 3	28
97	"			8.3	15
98	"			8 3	21
99	"			8.3	24
100	"			8 3	20
101	Hyperglycemic	1 0	1.50	8 3	32
102	"	1.3	1.50	8 3	22
103	"	1 0	1 00	8 3	39
104	"	1.0	1 00	8 3	40
105	"	1.3	1 50	8.3	46
Fasted, mean ± s E.†				9 2	48 1 ± 2.7
				8 3	22.4 ± 1 7
Hyperglycemic, mean ± s.E.†				9 2	85.0 ± 5.6
				8.3	35.8 ± 4.1

* See foot-notes to Table I.

† The value of t for the difference between the means at pH 9.2 of fasted and hyperglycemic rats is 6.61, with P = less than 0.01; at pH 8.3 t = 3.38, with P = 0.01.

rats given glucose by stomach tube when the test was performed on the dilute homogenates. However, when the concentrated homogenates from phlorhizinized animals receiving glucose were tested, kidney phosphatase activity remained inhibited.

TABLE III

Phosphatase Activity of Rat Kidney Extracts, at pH 5.0 and 9.2, before and after Addition of Phlorhizin to Final Concentration of 0.01 and 0.001 M

The results are expressed in mg. of P per hour.

Rat No.	Original phosphatase activity		Phosphatase activity after addition of phlorhizin			
			0.01 M phlorhizin		0.001 M phlorhizin	
	pH 5 0	pH 9.2	pH 5.0	pH 9.2	pH 5 0	pH 9.2
41	8.9	53	6.3	4.0		
42	6.0	29	4.0	1.5		
43	3.8	21	1.6	3.3		
44	5.0	24			3 4	11.5
45	4.1	15			3.4	4.2
Mean	5 56	28.4	3.97	2.93	3.40	7.85
% inhibition of phosphatase*	40	91	25	62		

* Corresponding values for phosphatase activity in the absence and presence of phlorhizin were used to calculate these average values for per cent inhibition.

TABLE IV

Acid and Alkaline Phosphatase Activity, at pH 5.0 and pH 9 2, of Kidneys from Normal and Phlorhizinized Rats

Rat No	State	Phosphatase activity	
		pH 5 0	pH 9 2
		mg. P per hr.	*mg. P per hr.*
35	Normal (fed)	0.62	8.7
36	" "	1.21	10 3
37	" "	0.81	8 5
94	" "	1.50	
95	" "	0.81	
38	Phlorhizinized*	0.69	6 9
39	"	0.29	3.4
40	"	0 75	6 1
Normal, mean ± s.e.†		0.99 ± 0.16	9.17 ± 0.57
Phlorhizinized, mean ± s.e †.		0 58 ± 0.14	5.47 ± 1.06

* Phlorhizin in oil administered subcutaneously for 3 days The dose was 140 mg. per kilo per day. Urine D:N ratios of the phlorhizinized rats averaged 4.3:1.

† In the case of the mean values for acid phosphatase, $t = 2.93$ and $P =$ less than 0.02, while for the alkaline phosphatase means, $t = 2.78$ and $P =$ less than 0.05.

The data in Table V suggest that the concentration of adenosine triphosphate does not play a determining rôle in the phenomenon of renal threshold for glucose. There was no difference in the ATP content of

TABLE V

Adenosine Triphosphate Content per 100 Gm., Wet Weight, of Kidney Tissue in 24 Hour-Fasted, Hyperglycemic (Given 1 Gm. of Glucose in 50 Per Cent Solution), and Phlorhizinized Rats

Rat No.	State	Time of analysis*	Adenosine triphosphate content†
		hrs	*mg. P per 100 gm.*
28	Fasted		3.7
29	"		9.1
50	"		5.7
51	"		7.7
52	"		6.5
53	"		5.8
54	"		5.5
55	"		11.0
56	"		6.5
57	"		10.3
58	"		10.1
59	"		7.9
60	"		8.5
61	Hyperglycemic	2.00	9.8
62	"	2.00	5.6
63	"	1.75	9.7
64	"	2.00	10.0
65	"	2.00	8.1
66	"	1.00	3.9
67	"	2.00	11.4
68	"	1.00	4.8
69	"	1.00	6.3
70	"	1.00	5.4
39	Phlorhizinized‡		7.1
40	"		6.3
71	"		11.1
72	"		11.8
73	"		11.5
74	"		6.4
75	"		15.7
76	"		6.8

Fasted, mean ± s.e.	7.56 ± 0.61
Hyperglycemic, mean ± s.e.	7.50 ± 0.83
Phlorhizinized. " ± "	9.59 ± 1.22

* Interval of time between the dose of glucose and nephrectomy.

† Expressed as mg of P liberated per 100 gm., wet weight, of tissue by 7 minutes of hydrolysis in 1 N H_2SO_4 at 100°.

‡ See foot-notes to Table IV.

kidneys from fasted and hyperglycemic rats, while ATP of kidney appeared to be slightly increased in the phlorhizinized rats.

Table VI

Phosphorylation and Oxygen Consumption in Kidney Homogenates from 24 Hour-Fasted and Hyperglycemic Rats

Rat No.	State	Glucose dosage*	Time of analysis†	Phosphorylation‡	Q_{O_2}§
		gm.	*hrs.*	*mg. P per 30 min*	*microliters per mg. per hr.*
77	Fasted			−5.0	3.0
78	"			−2.1	5.9
79	"			−1.5	4.5
80	"			−1.5	2.6
81	"			−1.8	2.3
82	"			−0.4	3.2
83	"			0.0	5.4
84	"			−1.1	6.4
85	"			−0.4	5.1
86	"			−0.2	4.2
87	"			−0.7	3.0
88	Hyperglycemic	1.75	1.25	−2.0	2.7
89	"	1.25	1.00	−1.0	3.6
90	"	1.25	1.00	1.1	3.5
91	"	1.00	1.00	3.1	4.3
92	"	1.00	1.00	0.0	5.7
Fasted, mean ± s.e.‖.				−1.34 ± 0.42	4.14 ± 0 45
Hyperglycemic, mean ± s e.‖.				0.24 ± 0.88	3.95 ± 0.50

* Total amount of glucose, administered by stomach tube in a 50 per cent solution

† Interval of time between the dose of glucose and nephrectomy.

‡ Expressed as decrease (negative sign) of inorganic phosphate in mg per gm , wet weight, of kidney tissue per 30 minutes at 38 3° in an atmosphere of pure oxygen

§ Oxygen consumption per wet weight of tissue.

‖ The value of t for the difference in means for phosphorylation in the fasted and hyperglycemic rats is 3 02, with $P =$ less than 0 01

Table VII

Phosphorylation and Oxygen Consumption in Rat Kidney Homogenates before and after Addition of Phosphatase

Rat No.	Phosphatase added*	Phosphorylation†	Q_{O_2}†
	mg	*mg P per 30 min.*	*microliters per mg. per hr.*
86	0.0	−0.20	4.2
	2.0	0 55	2.3
87	0.0	−0.70	3.0
	1.0	0.00	2.5
93	0.0	−0.63	4.2
	1.0	−0.45	2.4
	2.0	−0.25	2.3

* A solution containing 10 mg. of phosphatase powder (20) per ml. of 0 01 M NaOH was used. 1 mg. of this phosphatase preparation produced a 62 per cent increase in the alkaline phosphatase activity of the original homogenate.

† See foot-notes to Table VI.

Under the conditions of our experiments (Table VI), the average decrease of inorganic phosphate (as a measure of the phosphorylation process (16)) in fasted rat kidney homogenates was 1.3 mg. of P per gm. of tissue per 30 minutes, while in three out of five hyperglycemia experiments either no decrease or an increase in the inorganic phosphate was found. This finding could suggest that there was some inhibition of phosphorylation in the kidney homogenates from the hyperglycemic rats. The oxygen consumption of the kidney homogenates from the hyperglycemic animals was not appreciably lower than that from the fasted animals (Table VI).

Since in hyperglycemia there was an increase in phosphatase activity (Tables I and II) which might be responsible for the above apparent decrease in phosphorylation, various unsuccessful attempts were made to inhibit specifically the phosphatase activity without simultaneous inhibition of the phosphorylation process. The effect of increasing the phosphatase activity by the addition to kidney homogenates from normal fasted rats of an active preparation of beef kidney phosphatase (20) was, therefore, studied. In such experiments, reported in Table VII, a marked inhibition of both phosphorylation and oxygen consumption was demonstrated following the addition of phosphatase.

DISCUSSION

In our experiments the phosphatase mechanism has been definitely implicated in the renal reabsorption of glucose. However, we have been unable to exclude the possibility that the phosphorylation process is also involved. It is indeed likely that a type of homeostasis may exist between phosphorylation and dephosphorylation. A separation of the two processes was not possible in our experiments which suggest that an increase in phosphatase activity may produce a relative inhibition of the phosphorylating system.

A consideration of our experimental results, which indicate that alimentary hyperglycemia induces an increase in kidney phosphatase activity, leads us to propose the following hypothesis. The renal threshold for glucose is at least in part an expression of the limit to which phosphatase activity can be raised. Beyond this limit, tubular reabsorption is incomplete, and spillage of sugar into urine takes place. In this view, renal threshold for glucose (and probably for other threshold urinary constituents) becomes a dynamic function, under enzymic control.

Our demonstration that phlorhizin inhibits phosphatase activity both *in vitro* and *in vivo* lends support to the above hypothesis of the rôle of phosphatase in glycosuria. Our experiments with phlorhizin are consonant with Lundsgaard's original concept (2) of the action of phlorhizin, and go materially beyond the findings of Beck (4) in this connection. We believe

that two factors are largely responsible for the present successful demonstration of the effect of phlorhizin on both acid and alkaline phosphatase: (a) the preparation of more active extracts of kidney than those obtained by Beck (4), and (b) the use of concentrated kidney homogenates. Even in the case of the latter, in which phosphatase activities are relatively low, they are of similar or higher magnitude than those obtained by Beck.

The hypothesis which has been proposed for the reabsorption of glucose from the glomerular filtrate is consonant with current views upon the homeostatic control of blood and tissue sugar levels (21), and may thus have general applicability. Pertinent in this connection is the finding (22) that in glycogen disease the increased deposition of glycogen, accompanied by hypoglycemia, is associated with decreased alkaline phosphatase activity of the liver. Thus, when liver phosphatase activity is low, blood sugar cannot be produced in adequate amount, although glycogen is available. In an analogous way, when kidney phosphatase activity is insufficient, an adequate transfer of glucose to blood from the glomerular filtrate is not possible. It is also of interest that Bodansky (19) has reported an increase in serum alkaline phosphatase activity after the administration of carbohydrate.

The increase in phosphatase activity following induced hyperglycemia is of interest in itself. It is striking that in relatively brief periods of time such increases in enzyme activity could occur. At present no satisfactory explanation for this phenomenon is available.

SUMMARY

A modified method, by which active extracts are obtained, has been developed for the quantitative measurement of kidney phosphatase activity.

The following findings have been made.

1. Induced alimentary hyperglycemia was found to be accompanied by increased acid and alkaline kidney phosphatase activities. The increases were, respectively, 55 and 70 per cent.

2. The inhibition of both acid and alkaline kidney phosphatase activities by phlorhizin has been demonstrated *in vivo* as well as *in vitro*. 0.01 M phlorhizin produced an inhibition of 40 per cent in acid and 91 per cent in alkaline phosphatase activity.

3. The adenosine triphosphate content was of the same magnitude in the kidney tissue from fasted and hyperglycemic rats.

4. An inhibitory effect upon phosphorylation and oxygen consumption, due to increased dephosphorylation, was demonstrated in experiments in which phosphatase activity was increased by means of the addition of a preparation of the enzyme to kidney homogenates.
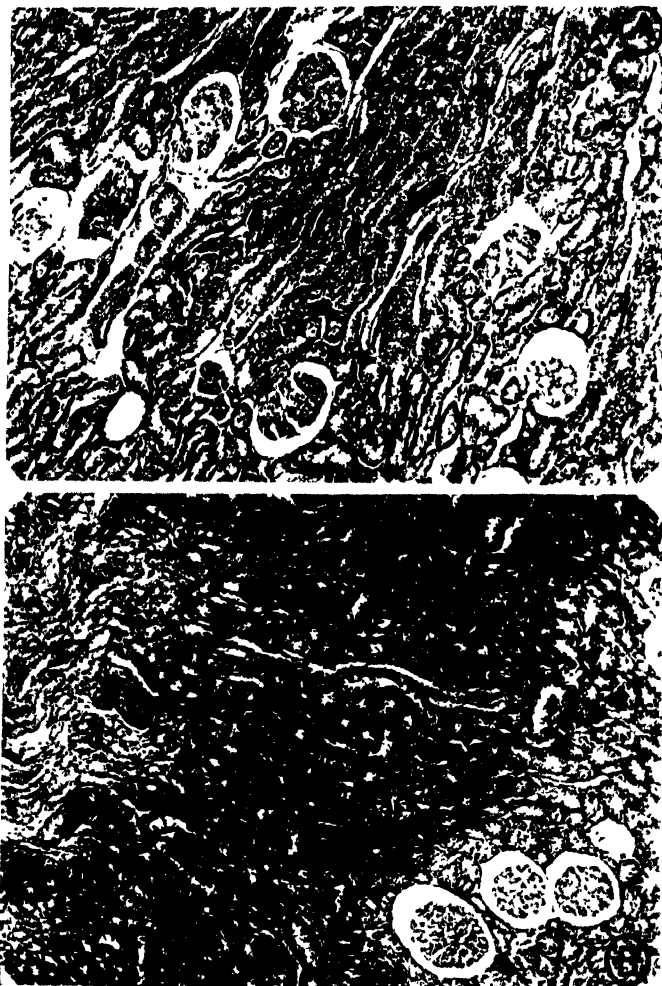
(Marsh and Drabkin Phosphatase and glycosuria)

The probable significance of the findings has been discussed with reference to the rôle of phosphatase in the tubular reabsorption of glucose. It has been postulated that the phenomenon of renal threshold for glucose is at least in part an expression of the limit to which kidney phosphatase activity can be raised.

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EXPLANATION OF PLATE 1

FIG. 1. Kidney sections from fasted (*A*) and hyperglycemic (*B*) rats, stained by Gomori's technique (5) for acid phosphatase activity; incubation period 7 hours at 37.5°. × 65.

IMMUNOLOGICAL REACTIONS BETWEEN FILMS OF ANTIGEN AND ANTIBODY MOLECULES

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The fundamental nature of the reactions between antigen and antibody molecules is far from being understood in spite of a tremendous amount of accumulated experimental data. A number of theories have been proposed on the basis of the available experimental evidence. Some of the facts, however, which were used in support of a given theory could as well have been used to justify a different point of view.

The immunological theories proposed so far can roughly be classified into two main groups which, at first sight, would seem incompatible with each other. Bordet, a representative of the first group, maintained that antigen and antibody molecules combine in continuous proportions. More recent authors, who belong to the second group, assume that immunological reactions are nothing more than ordinary chemical reactions, and that combinations, therefore, occur in definite multiple proportions. It will become apparent from the present immunological investigation that it seems difficult if not impossible to harmonize the presented facts with this latter point of view.

The aim of this study was to investigate the conditions under which an antigen, when it is present as a monomolecular layer on a metal slide, will attach specifically homologous antibody molecules. It can be shown readily that a film of antigen deposited on a metal slide will adsorb a definite thickness of homologous antibody molecules when brought into contact with a solution of the latter (1). Thus a specific force of some kind is easily demonstrated. It was only logical to raise the question of just how far such a force would extend. The possible use of inert film barriers whose exact thickness could be measured readily appeared to offer a unique way of investigating this question. A most striking phenomenon was quickly brought to light. It was found that up to a certain thickness the intervening screen or barrier did not altogether suppress a specific interaction between antigenic films and antibody molecules. From these new facts it would appear that the old colloidal view of Bordet is justified in part for the interpretation of the mechanism of immunological reactions. The proponents of the chemical theories may have gone too far in the opposite direction, and it may turn out in the end that both view-points can be looked upon as limiting cases of a more general theory.

Procedure

The experimental procedure consisted in spreading films of antigen at an air-water interphase and then transferring them on metal slides according to the method of Blodgett and Langmuir (2). When a solution of homologous antiserum was brought into immediate contact with the slide, the thickness of the adsorbed layer, measured after washing and drying, corresponded to the amount of antibody molecules immobilized on the antigenic films. The main object of this study was to investigate the effect on the immunological reaction of intervening barriers of inert material deposited on the antigenic films.

Since all our deductions are based on measurements of thickness of films and adsorbed layers, the technique involved in this type of measurement will be first reviewed.

Film thicknesses were determined optically. They were obtained by measuring the change that takes place in the ellipticity of polarized light reflected from a polished stainless steel slide after it had been coated with films under investigation. This was accomplished with the help of an apparatus recently described, called the "ellipsometer" (3), a differential instrument based on the half shadow principle. The half shadow is obtained by covering a clean polished metal slide with one, two, or more reference films, and the lower half of the slide with two additional layers. The reference films used were either barium stearate or octadecylamine. Since the difference in thickness of the films covering the upper and lower halves of the slide is 49 A, the difference in ellipticity of the light reflected from these two half fields is such that both halves of the slide appear of unequal intensity when viewed through a quarter wave-length plate and analyzer properly oriented. The measurement consists in rotating the analyzer until both halves of the slide are matched. This position of the analyzer gives the zero reading for the particular slide. All slides are covered with such an optical gage and the zero reading is taken before films of unknown thickness can be measured. These need to be deposited on the central part of the slide only, where the dividing line between the upper and the lower part is located. This added layer changes the ellipticity and orientation of the ellipses corresponding to both half fields, which then become unequal in intensity. Equal intensity can be restored by rotating the analyzer, whose angle of rotation is a measure of the thickness of the film. The technique is comparable to that used in polarimetry.

For routine measurements calibration curves, as previously described (3), were found most useful. Increments in thickness are characterized by rotations in one direction and decrements by rotation in the opposite direction. The sensitivity of the apparatus permits determination of an

transferred to the metal slides. Surface pressure was measured by the vertical pull method (5). The antigenic films could be transferred to four or five slides at one time by means of a special stand, a photograph of which can be seen in Fig. 1. The knob K, which determines the vertical motion of the slides, carries a scale to facilitate the control of the depth to which the slides are immersed. One monolayer of antigenic film can be transferred either by immersing the slide into the tray before the protein has been spread (the symbol ↑ is used to denote this operation) or by removing the film from the surface of the trough after the slide has been immersed (symbol ↓). It was found easy to transfer two monolayers of most proteins. However, it was difficult to deposit more than two layers
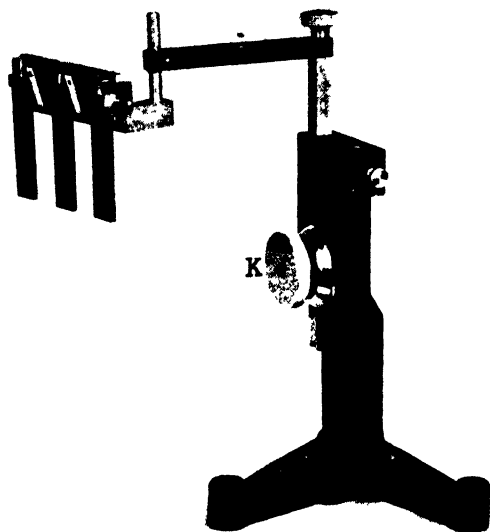
FIG 1   Stand for film transfer on five slides simultaneously

on an optical gage of barium stearate. In the case of bovine albumin, for instance, it was impossible to transfer three or more double layers of the film. However, it was found that if the slides covered with an optical gage of barium stearate were first conditioned with a solution of uranyl acetate (6) many double layers could be deposited without difficulty. This is accounted for by the fact that the first anchored molecules were so strongly polarized that they polarized in turn the molecules deposited on top, and so on along the successive layers. This polarization increases the bonding energy of the successive layers, which becomes strong enough to prevent the top layers from slipping back onto the water surface when the slides are taken out of the trough. Thickness measurements demonstrated

optical thickness within ±0.3 A.   The absolute thickness depends on the knowledge of the refractive index of the films.   Two indices are needed if the films are anisotropic.   In all the measurements reported in this article the assumption was made that films and adsorbed layers have the same refractive index as barium stearate ($n_D^{25} = 1.495$).   The difference between the values of the refractive index of proteins and barium stearate is not large enough to introduce any serious error.   In any case it would not affect the relative thickness of the layers upon which the present considerations are based.   It might be said that for an angle of incidence of 69.2°, at which the measurements were made, and for the range of thicknesses considered, the added layers affect mainly the phase difference of the components OP vibrating in the plane of incidence and OS vibrating perpendicular to the plane of incidence (3) and to a lesser degree their relative magnitude.   For a given angle of incidence the phase shift Δ is given by the equation $\Delta = -A(1 - (1/n^2))l$, where A is a constant, n the refractive index, and l the thickness of the layer (4).   It is therefore apparent that a change in the refractive index from 1.50 to 1.55 will bring about a difference of 5 per cent only in the corresponding phase shift and consequently in the thickness of the films.

The preparation of the optical gages on the metal slides has been discussed elsewhere (3).

The assumption made in the measurement of deposited layers is that the thickness is the same on the upper and lower parts of the slide.   This seems always to be the case with films transferred onto a slide from a liquid interphase.   On the other hand, when molecules are adsorbed on a slide, the amount adsorbed on one monolayer of reference film is not necessarily the same as on three layers.   To test this possibility many experiments were carried out with optical gages made of one and three, as well as two and four, and three and five, layers of reference films.   In all cases the results obtained were independent of the number of layers of the optical gage, except possibly in one case which is discussed later. It is of interest to note that slides covered with barium stearate are dry when emerging from the tray, whereas they are wet when coated with octadecylamine.

Spreading of Antigen Films—Films of antigen were formed by spreading a drop ($\simeq 0.02$ cc.) of an aqueous solution of the antigen on the surface of a clean Adam-Langmuir trough filled with twice distilled water.   Two troughs made of Plexiglas were used, one 50 × 14 cm. and the other 30 × 13 cm.

The concentration of the antigen solution was such as to allow the covering of three-fourths of the surface of the trough at zero pressure. 3 minutes after spreading, the films were compressed to 8 or 9 dynes and

that the antigenic films consisted of completely unfolded molecules, since the thickness per monolayer was found consistently between 8 and 9 A after transfer under 8 dynes of pressure.

The conditioning solution of uranyl acetate (7) was made up of equal parts, mixed just before use, of a 10^{-3} M solution of uranyl acetate and a veronal buffer, 0.02 M, pH about 7. The pH of the mixture was about 6.6. The plates were wetted by such conditioning as long as the pH stayed within 5.5 to 7.5. The conditioning was accomplished by covering the slide for 3 minutes with approximately 1 cc. of the solution, after which the plate was washed with redistilled water and dried. The increase in optical thickness resulting from conditioning was $\simeq 8$ A.

Deposition of Screens—The screens consisted of layers of barium stearate, octadecylamine, protein multilayers, and Formvar, which according to the manufacturer is a polyvinyl formal resin with an average molecular weight of 13,000 to 15,000. Barium stearate and octadecylamine screens were formed in the same way as the optical gages. It is of interest to mention that when a double layer of barium stearate was deposited on one or two monolayers of protein the slide came out wet, a film of water being squeezed in between the two layers. After drying, the slide was hydrophobic. On the other hand, if the slide had been conditioned before the transfer of the protein films, it came out dry after the deposition of the double layer of barium stearate. The deposition of the screen was made at room temperature. It was observed that barium stearate screens could not be deposited regularly on a double layer of egg albumin at a temperature above 25°, but the deposition proceeded easily at 20°. This temperature effect depends partly on the protein, since stearate screens could be deposited above 25° on a double layer of bovine albumin.

The *protein screens* were made in the manner described for the transfer of antigenic films. One essential condition in the choice of the substance is that there is no cross-reaction with the particular antiserum.

Formvar screens were formed by smearing a drop of an ethylene dichloride solution on the slide, which was then allowed to dry in a vertical position. By using drops of the same size it was found that the thickness of the screen was roughly proportional to the concentration used. For instance, a solution of 0.1 per cent gave a screen of about 150 A. In a few cases the Formvar films were made on clean microscope slides. If, after evaporation of the solvent, the slides were quickly plunged into a dish filled with water, the films could be detached and floated on the surface. They could then be transferred on protein-coated metal slides in two ways, either by lowering the slide face down into the surface or by dipping the slide into the dish under the film and lifting it. In the first method the slides were dry when the film was picked up; in the second they were wet

with a layer of water between slide and Formvar film. After drying, the slide became hydrophobic.

Reaction with Antisera—All sera were diluted 1:10 with phosphate buffer of pH 7.05 (0.825 gm. of NaOH and 3.45 gm. of KH_2PO_4 per liter), containing 0.9 per cent NaCl. A drop of such diluted serum was smeared on the slides to be tested (with or without screen) and left there 10 minutes before washing. For the adsorption period the slides were placed in horizontal test-tubes containing a piece of wet absorbing cotton to prevent the drying of the slides. At room temperature the reaction proceeded extremely rapidly and was nearly completed in 2 to 3 minutes.

Washing—The slides were washed with water after the deposition of the antigenic layers. They were washed after adsorption of the serum with the saline phosphate solution used for the dilution of the serum and then with redistilled water.

Reproducibility of the results was found to depend in some measure on uniform washing. Therefore in washing, the slides were held in clamps attached at about 20° from the vertical position to a movable rack and could be brought into contact in turn with the tips of two special 15 cc. pipettes, the design of which can be seen in Fig. 2. The flattened end of the pipettes was 2 cm. $\times$ 0.1 cm. It required about 3 seconds for them to empty. As it appears from the drawing, the pipettes were automatically filled each time to the same level. The temperature of the washing solutions was found to be of no importance as long as it did not go much above 20°. The bottles containing the stock solutions were kept in a tank cooled with running water or ice.

Systems Investigated—Experiments were carried out with the following antigens: crystalline egg albumin, crystalline bovine albumin, polysaccharide from pneumococcus type III, and crystalline ferritin and apoferritin. Rabbit immune sera were used exclusively.[1]

Reproducibility of Results—Generally the amount of specifically adsorbed antibody, with or without screen, could be reproduced within 10 per cent. Thus when thick layers of antibodies were adsorbed, the variations were well above the limit of sensitivity of the ellipsometer. The causes of the variations have not as yet all been traced. For instance, it was established in the case of films of bovine albumin that the amount of adsorbed antibody was consistently larger by 10 per cent when the films had been spread on

water redistilled from a quartz still than when spread on distilled water
from a commercial still, tin-lined.

Most of the data are presented graphically. The points of all the
curves represent average values obtained from five to fifteen similar experi-
ments, all falling within 10 per cent. When the scattering was larger,
single experiments have been indicated on the graph. Finally, all the
data reported have been obtained with a single antiserum for each antigen.
This was done to eliminate individual variation from serum to serum. It
should be said that numerous experiments were made with sera from
different rabbits which gave comparable results.
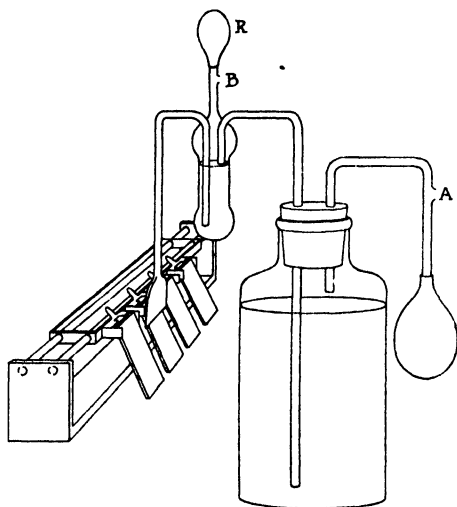
FIG. 2. Constant volume delivery pipette. Hole *A* is closed when filling the
pipette; *B* is closed when emptying the pipette with the aid of rubber bulb *R*.

Egg Albumin-Anti-Egg Albumin Rabbit Serum—Many experiments with
this system were published in a preceding article (1) which showed that
an unfolded film of egg albumin was still capable of reacting specifically
with an immune serum. Data obtained in dealing with the screening
action of barium stearate have been condensed in Table I. Experiments
were carried out with one double layer of egg albumin, although the num-
ber of layers of this spread antigen had no influence on the amount of
antibody specifically adsorbed during subsequent treatment with an im-
mune serum. The mode of deposition of the layers, ↓ or ↑, as well as the
conditioning of the slide with uranyl acetate solution did not alter the
results.

A study of Table I reveals that a specific fixation of antibody occurs,

notwithstanding an intervening screen of four layers of barium stearate. It also shows that a heterologous serum deposited directly on the antigenic film or on a screen of one monolayer of barium stearate does not bring about any non-specific increment in thickness. However, there occurred a small non-specific fixation which increased with each additional layer of stearate until it reached the value of 15 A, which is also the increment observed upon treatment of a slide covered with a stearate layer without any underlying protein film. In this connection the following generalization can be formulated:

A slide covered with barium stearate layers exhibits an increase of 15 to 20 A after treatment with any dilute serum. No increase is observed if previous to the treatment a double layer of a protein has been transferred to the slide. In other words, 15 to 20 A are the maximum non-specific

TABLE I

Screening Action of Barium Stearate on One Double Layer of Egg Albumin or Bovine Albumin

Screen, No of monolayers of barium stearate, 24 4 A per layer	Increase in Ångstrom units			
	Egg albumin film after treatment with anti-egg albumin serum	Bovine albumin film after treatment with anti-egg albumin serum	Bovine albumin film after treatment with antibovine albumin serum	Egg albumin film after treatment with antibovine albumin serum
0	26	0	65	0
1	21	0	39	0
2	21	5	33	0
3	21	7	25	0
4	17	11	23	6
6	16	15	10	10

thickness of film which can be adsorbed directly on a slide covered with layers of barium stearate.

A curious observation was made in studying the effect of underlying layers on the reactivity of egg albumin films, an observation which has its counterpart, as will be seen later, in the bovine albumin system. The thickness of specifically adsorbed anti-egg antibody was diminished by half (12 A instead of 26 A) if the monolayer of egg albumin was deposited on a slide already covered with a monolayer of bovine albumin The same specific increment of 12 A was observed if the order of deposition of the transferred layers was altered.

Experiments were also made in which egg albumin molecules were adsorbed directly onto the slide instead of being transferred from a water interphase. This was done simply by smearing a drop of dilute solution of this protein on a slide coated with an optical gage. The adsorption

took place very rapidly, since the same results were obtained whether the solution was kept 5 seconds or 3 minutes on the slide. The slides were washed with water only, since the phosphate-saline buffer removed the adsorbed molecules. The thickness of the adsorbed layer of egg albumin was 20 A, and there was an average specific increment of 70 A, with considerable scattering, following treatment with an immune serum. In a series of twenty experiments the values found fell within the range of 55 to 94 A. When the adsorption of the egg albumin molecules took place on a slide conditioned with uranyl acetate, the apparent thickness of the adsorbed antigen was the same but the average increment of antibodies was 100 A, the scattering covering the range 82 to 120 A in ten experiments. No increase was observed on treatment with an antibovine albumin serum. With an intervening screen of one double layer of barium stearate the increment in antibodies dropped to $\simeq$30 A.

These results demonstrate that unleafing the egg albumin molecules at the water interphase reduces considerably the subsequent interaction with antibodies. The thickness *per se* of the egg albumin layers is not an influential factor, since many piled up layers of egg albumin did not exhibit the reactivity of the adsorbed molecules.

Bovine Albumin Films-Antibovine Albumin Rabbit Serum—The bulk of our data deals with this system because of an interesting property characteristic of this system which permitted the extension of the scope of the investigation. It was found that the thickness of the specifically adsorbed antibody layers increased linearly with the number of underlying films of bovine albumin up to four double layers. This is in direct contrast to the results found in the case of egg albumin and other systems such as metakentrin-antimetakentrin (8). As in the egg albumin system, the mode of deposition of one monolayer, $\downarrow$ or $\uparrow$, had no influence on its reactivity. The uranyl acetate conditioning of a slide already coated with multilayers of bovine albumin decreased markedly the amount of antibody which could subsequently be adsorbed. As already mentioned, conditioning increased the optical thickness by 8 to 10 A. When a few monolayers of antigenic film are transferred to a slide, the uranyl acetate treatment can be applied at different stages of the deposition of the monolayers. The influence of the various possibilities on the immunological reaction has been summarized in the curves of Fig. 3. In these experiments, all slides were covered with an optical gage of two and four monolayers of barium stearate and then conditioned with uranyl acetate. Following this they were coated either with N monolayers of bovine albumin (Curve A), or with N monolayers of bovine albumin with one conditioning treatment between successive monolayers (Curve B), or with N monolayers of bovine albumin and one conditioning applied on top of the monolayer last de-

posited (Curve C), or with N monolayers of bovine albumin with one conditioning treatment between successive monolayers and one last conditioning on top of the layer last deposited (Curve D). The most striking effect is the decrease from 230 to 125 A in the antibody adsorbed, brought about by one conditioning of a slide coated with eight successive monolayers (Curves A and C), whereas the reactivity of one double layer is not markedly impaired by conditioning.
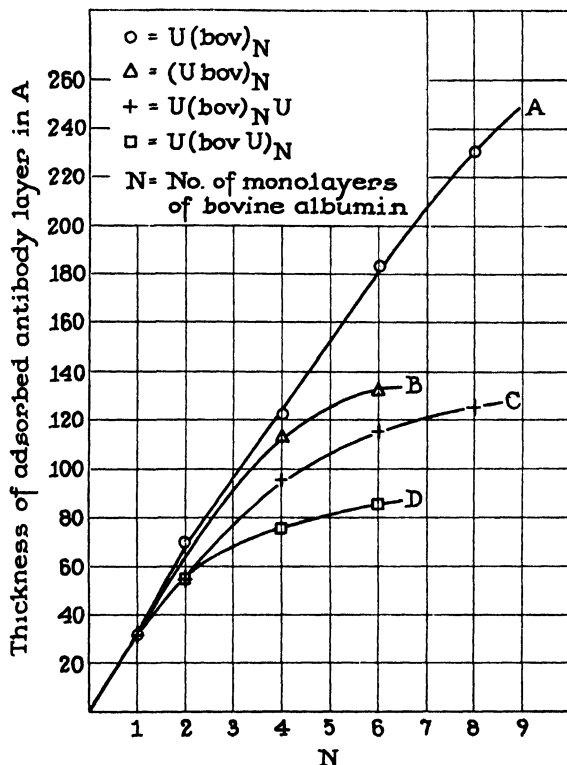
FIG 3 Effect on the amount of specifically adsorbed antibody after the deposited antigenic layers have been conditioned with uranyl acetate. Bov stands for one bovine albumin monolayer and U for one conditioning treatment.

The screening action of layers of barium stearate on one, two, and three double layers of bovine albumin is represented by Curves A, B, and C, respectively, in Fig. 4[2] which demonstrate that there is no appreciable specific adsorption when a screen of three double layers of stearate (146 A)

[2] Preliminary results published in a previous note (6), obtained under slightly different conditions, gave somewhat smaller amounts of specifically adsorbed antibody.

covers one double layer of bovine albumin (Curve A), whereas there is an increment of about 45 A when a similar screen or one of octadecylamine or
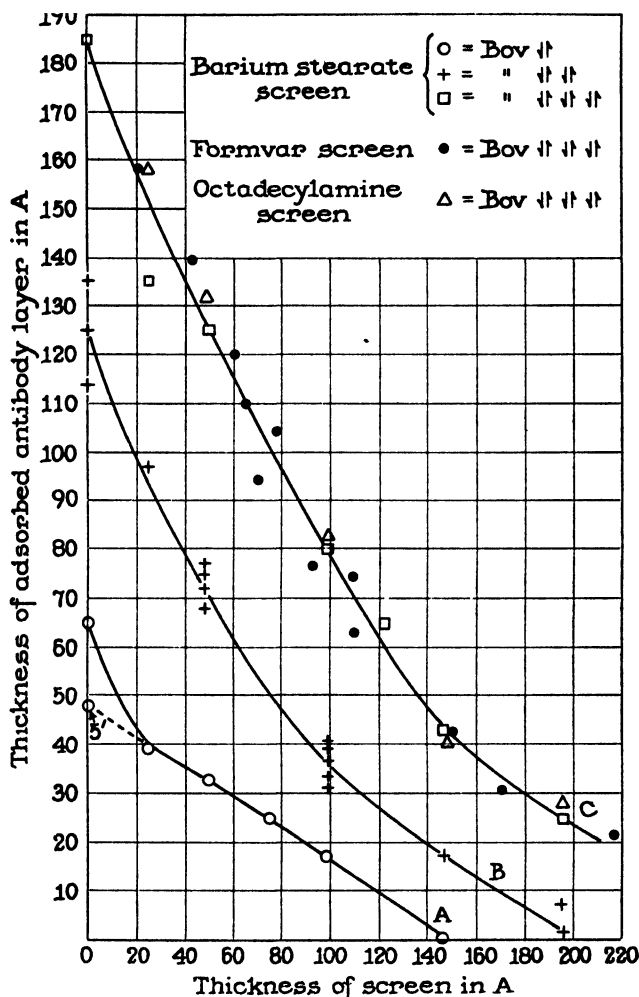
FIG. 4. Screening action of barium stearate, octadecylamine, and Formvar films on one, two, and three double layers of bovine albumin (Curves A, B, and C) deposited on a conditioned optical gage of two and four layers of barium stearate. Thicknesses of the layers of adsorbed antibody are true specific increments. Non-specific increases varying from 0 to 15 A, depending on the thickness of the screen, were subtracted from the experimental values. The symbols Bov ⇅, Bov ⇅ ⇅, Bov ⇅ ⇅ ⇅ stand for one, two, and three deposited double layers of bovine albumin.

Formvar of the same thickness is deposited on three double layers of albumin (Curve C).

The amount of scattering among the data does not allow precise curves to be drawn, but as a first approximation it can be said that the amount of adsorbed antibody decreases linearly with the increase in thickness of the screen. It is evident from Curve C of Fig. 4 that the efficiency of the three kinds of screens tested depended mainly on their thickness and very little if at all on their chemical nature. It should be remembered, however, that the fabric of all three screens is constituted in the main by C—C and C—H bonds. We plan to investigate the screening action of metal films whose conductive properties put them in an entirely different category.

TABLE II

Screening Effect of Monolayers of Egg Albumin on Layers of Bovine Albumin

No. of monolayers of bovine albumin	2	2	2	2	2	2	4	6	6
Screen; No. of egg albumin monolayers, 9 A per layer		2	4	6	8	10	4	6	10
Increase after adsorption with antibovine albumin serum for 5 min., A	56	41	29	17	9	0	71	106	76

TABLE III

Influence of Underlying Layers of Egg Albumin on Reactivity of Bovine Albumin Layers

Increase in Ångström units, after adsorption of antibovine albumin serum for 5 min

$O{\downarrow}{\uparrow}$	$B{\uparrow}$	$B{\downarrow}{\uparrow}$	$O{\uparrow}B{\uparrow}$	$O{\downarrow}{\uparrow}B{\downarrow}{\uparrow}$	$O({\downarrow}{\uparrow})_3 B{\downarrow}{\uparrow}$	$O({\downarrow}{\uparrow})_3 B{\downarrow}{\uparrow}$	$O({\downarrow}{\uparrow})_4 B{\downarrow}{\uparrow}$	$B({\downarrow}{\uparrow})_3$
0	25	50	48	90	92	88	84	135

O and B stand for egg and bovine albumin, respectively. Symbols $\downarrow$ and $\uparrow$ indicate the mode of deposition of one layer. The values in this table are consistently smaller than those in Table I, since the time of adsorption was 5 minutes instead of 10 minutes.

The amount of adsorbed antibody with screens made of transferred egg albumin layers also diminishes linearly with the increase in thickness of the screen, as is shown in Table II.

All the data presented so far have been obtained with antigenic layers deposited on slides covered with a conditioned optical gage of two and four layers of stearate. It was observed that if bovine albumin layers were transferred to conditioned slides coated with one monolayer of egg albumin the subsequent increment of antibody was larger than when the egg albumin layer was not present. The results obtained with different possible combinations are presented in Table III. It appears from the data that an underlying egg albumin layer has an influence on the reactivity not only of the layer directly above but also on the next one, since the

amount adsorbed on $O \Uparrow B \Uparrow$ is nearly twice as large as that on $O \uparrow B \uparrow$ or $O \Uparrow B \uparrow$ (O = ovalbumin, B = bovine albumin).

Influence of Temperature on Adsorption—The thickness of the layers of antibody adsorbed within a given time is diminished by lowering the temperature. In 5 minutes the amount adsorbed at 5° was about 80 per cent that adsorbed at room temperature ($\simeq$25°), regardless of the pattern of the antigenic films. Comparable data were obtained with the systems $[B \Uparrow]$, $[B \Uparrow$ stearate $\Uparrow]$, and $[B(\Uparrow)_3]$. The data show that the main factor involved is the diffusion of the antibody molecules dissolved in the liquid phase, into the surface of the slide.

The influence of the time of contact of the immune serum with the screen was investigated. It was found that no additional increase in antibody took place after a period of 10 minutes. For instance, the same value of 72 A (specific increase) was observed after 10 minutes or after 40 minutes in the case of three double layers coated with a screen of Formvar 110 A thick.

Polysaccharide from Pneumococcus Type III and Antiserum—In this case, the antigen could not be spread at a water interphase but was easily adsorbed on a slide. Drops of a 0.04 to 0.02 per cent solution of polysaccharide were smeared for 3 minutes on slides which were then washed with water. The adsorbed layers were all about 5 A thick or less when adsorption took place on uranyl acetate-conditioned slides coated with an optical gage of stearate or octadecylamine. It was found that the nature of the gage, stearate or octadecylamine, on which the polysaccharide was adsorbed had a very great influence on the amount of adsorbed antibody.

When the polysaccharide was adsorbed on a gage of barium stearate, the thickness of the antibody layer subsequently immobilized was 120 to 130 A after treatment with an immune serum for 10 minutes. This value was not increased by lengthening the adsorption period to 1 hour. Occasionally the observed increases for a series of slides were consistently larger (170 to 180 A) or smaller (95 to 110 A). These variations seem to be caused by an impurity affecting the barium stearate gage and, hence, the structure of the adsorbed polysaccharide layer, since all slides prepared simultaneously exhibited increases within the same range of thickness. In other experiments, slides coated with a conditioned gage of barium stearate were covered with a double layer of egg albumin prior to the adsorption of the polysaccharide. The subsequent increase in adsorbed antibody was found consistently between 140 and 150 A. The influence of screens of barium stearate, octadecylamine, and Formvar on the amount of specifically adsorbed antibody was also investigated. Special precautions had to be taken when screens of Formvar were deposited on polysaccharide adsorbed on a barium stearate gage. These gages consisted
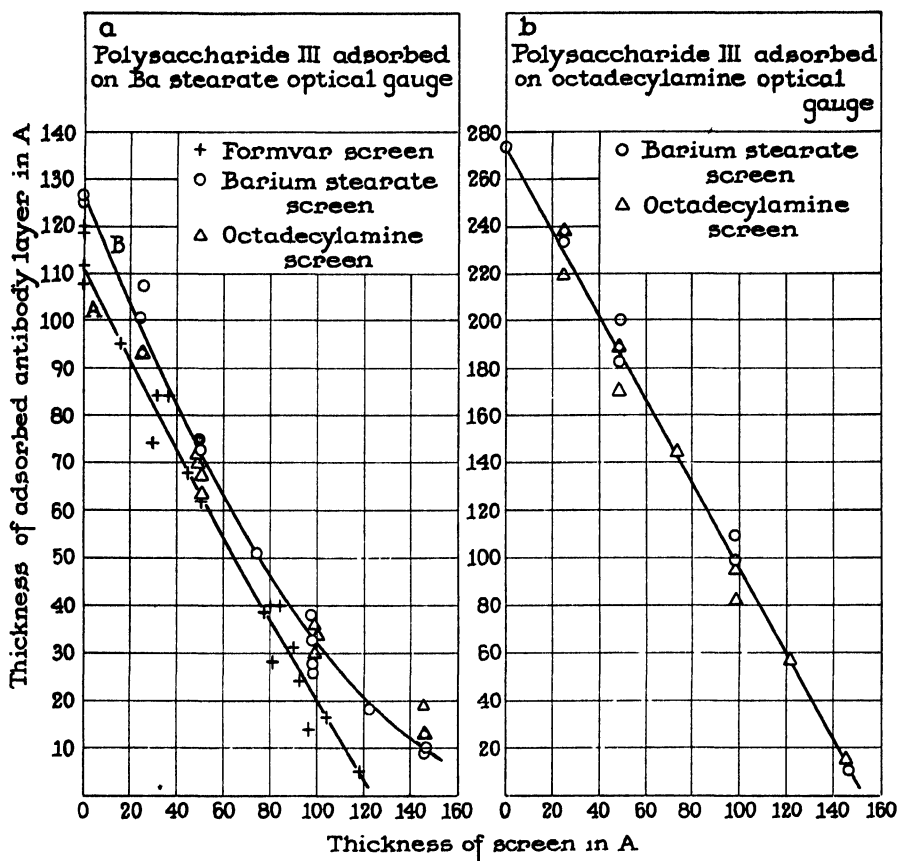
FIG. 5, *a*. Screening action of barium stearate, octadecylamine, and Formvar films on polysaccharide type III, adsorbed on a conditioned optical gage of three and five monolayers of barium stearate. Thicknesses of the layers of adsorbed antibody are true specific increments. Non-specific increases varying from 9 to 17 A, depending on the nature and thickness of the screen, were subtracted from the experimental values. They were obtained upon adsorption of the antiserum on the screens without underlying polysaccharide.

FIG. 5, *b*. Screening action of barium stearate and octadecylamine on polysaccharide type III, adsorbed on an optical gage of octadecylamine. Thicknesses of the layers of adsorbed antibody are true specific increments obtained after 10 minutes. Non-specific increments of 15 A were subtracted from the experimental values. The optical gage, consisting of three and five monolayers of octadecylamine, was conditioned twice with uranyl acetate, once after the deposition of the first monolayer and then after completion of the deposition of the gage.

mainly of barium stearate but also contained some free stearic acid which was dissolved by ethylene dichloride during the formation of the Formvar

barrier, thus causing an error in the determination of the screen thickness important in the range of thin screens only. To eliminate this error, the slides to be coated with Formvar screens were treated with ethylene dichloride prior to the adsorption of the polysaccharide type III. As is shown in Fig. 5, *a*, the amount of antibody immobilized when no screen was present was somewhat smaller when the gage had been treated with ethylene dichloride (Curve A) than when it had not been so treated (Curve B). The curves of Fig. 5, *a* also demonstrate that the screening action of the three types of barriers was essentially the same, except in the region of the thicker screens, when a specific adsorption was still observed with a screen of barium stearate or octadecylamine 146 A thick, while no such reaction took place with a Formvar screen of the same thickness.

When the polysaccharide was adsorbed on a gage of octadecylamine, the thickness of the layers of antibody was 280 A after 10 minutes, 600 A after 1 hour, and 700 to 800 A after 2 hours, a most amazing increase when it is considered that it is brought about by a layer of polysaccharide only 5 A thick. The optical gage of octadecylamine consisted of three and five monolayers, since in this case it was apparent that the amount of specifically adsorbed antibody was greater on the lower than on the upper part of the slide when a gage of only one and three monolayers of octadecylamine was used. When an optical gage of stearate was coated with one double layer of octadecylamine before the polysaccharide was adsorbed, a similarly large amount of antibody was immobilized, the reaction being even faster (500 A in 10 minutes). The curve in Fig. 5, *b* demonstrates that the screening action of barium stearate or octadecylamine on a layer of polysaccharide adsorbed on an octadecylamine gage is roughly the same, no specific adsorption of antibody being demonstrable for screens thicker than 150 to 160 A. The amount of antibody immobilized decreases linearly with the increase in thickness of the screen. The influence of a Formvar screen, however, seems in this case to follow a slightly different course, as is shown in Fig. 6, but the minimum thickness of the screen necessary to prevent a specific fixation of antibody has the same value of 120 to 130 A as that found when the polysaccharide was adsorbed on a barium stearate gage.

As already observed for the bovine albumin system, when there was a screen the maximum value of the thickness of the antibody layers adsorbed was obtained in about 10 minutes. For instance, when the polysaccharide was anchored on an octadecylamine gage and coated with a barrier of one monolayer of octadecylamine, the specific adsorbed layer of antibody observed after 10 or 60 minutes was 240 A thick.

Ferritin, Apoferritin-Antiferritin Serum—A few experiments were carried out with this system and the results are of sufficient interest to be

recorded. Both ferritin and apoferritin were crystalline samples of high degree of purity. These proteins spread with considerable difficulty on water and the thickness per monolayer was 25 A; in other words, the molecules were not completely unleafed. When treated with a dilute
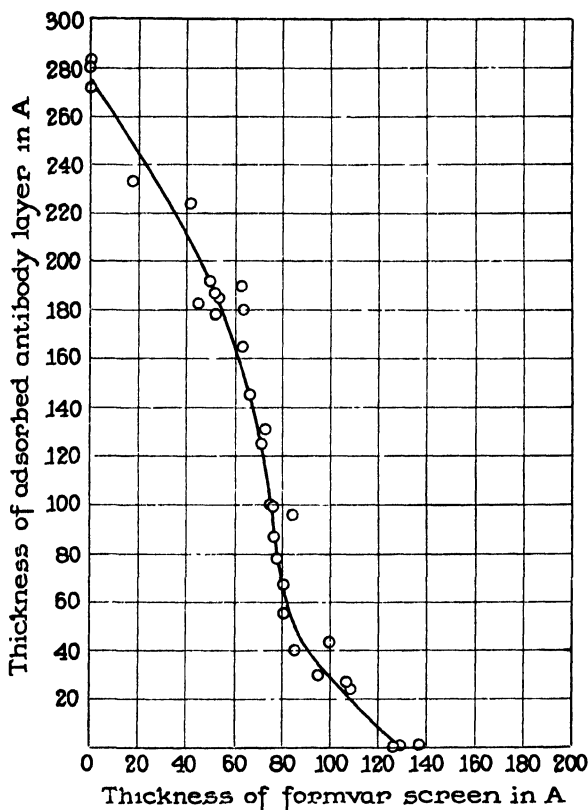
FIG 6. Screening action of Formvar films deposited on polysaccharide type III, adsorbed on an optical gage of octadecylamine Thicknesses of the layers of adsorbed antibody are true specific increments obtained after 10 minutes Non-specific increases of 13 A were subtracted from the experimental values. The optical gage of three and five monolayers was the same as that described for Fig. 5, b.

antiferritin serum, films from both proteins were capable of adsorbing specifically a layer 25 A thick.

Ferritin and apoferritin molecules could also be adsorbed on slides covered with a stearate gage, and the same increase of 25 A was observed after treatment with the antiserum. This increase was independent of the thickness of the adsorbed antigenic layer (from 30 to 70 A).

If, however, apoferritin was spread on a 1 per cent NaCl solution, the thickness of a double layer was 18 A, corresponding to a completely unfolded polypeptide chain of 9 A per layer. The films were found totally inactive. Activity could not be restored by increasing the thickness up to five such double layers by successive dipping. It is apparent that complete unfolding of the large protein molecule of apoferritin (mol. wt. 465,000) (9) destroys its immunological reactivity.

DISCUSSION

The main body of the data presented in the preceding section concerns the effect on immunological reactions of barriers interposed between films of antigens and homologous antibodies. Before these results are discussed, some remarks on the direct reaction occurring without intervening screens would appear advisable.

From an immunological point of view, films of apoferritin, egg albumin, and bovine albumin can be said to be characteristic of three different classes of protein films. Apoferritin molecules on complete spreading lose their reactivity. The necessary pattern for the immunological reaction therefore requires more than a two-dimensional fabric. It is to be expected that large molecules, which suffer more steric disturbances on unfolding, will be found in this category.

Egg albumin probably belongs to the most common type; unfolding does not destroy the immunological reactivity but reduces it. It was found that the same specific increment occurred after treatment with an immune serum whether the slide had been coated with one or many monolayers. In other words, a reactivity equal to that of the adsorbed molecules cannot be regained by building up successive monolayers.

In contrast, bovine albumin monolayers not only retain their reactivity but possess the remarkable property of additive reactivity. The amount of specifically immobilized antibody is directly proportional to the number of underlying layers, up to a certain number.

Great difficulty is therefore encountered in trying to explain such phenomena on the basis of the current chemical theories which axiomatically assume that the reaction occurs between definite groups of the antigen and definite groups of the antibody. If this were the case, how could "definite" groups of the antibody molecule react with definite groups of an antigen film buried under seven layers of the same antigen, 56 A deep (see Fig. 3)? Would steric hindrance not be an insurmountable barrier? It could, of course, be argued that during the course of the reaction the layers of the protein are sufficiently disrupted to permit close contact between these hypothetical groups. If the antibody molecules were really coming into contact with specific groups of the successive antigenic

layers of bovine albumin, then when the slide was covered with successive egg albumin monolayers the same phenomenon might be expected to occur upon treatment with homologous antiserum. On the contrary, in the latter case the amount of specifically adsorbed antibody is independent of the number of underlying egg albumin monolayers.

A further point worth considering in this connection is the absolute amount of antibody immobilized by the antigenic layers. It has been shown that a layer of antibody 230 A thick can be adsorbed on four double layers of bovine albumin. This thickness is greater than that to be expected from close packing of a single layer of molecules of rabbit antibody, unless they are adsorbed on end. Furthermore, the fact that a layer of antibody 700 A thick was adsorbed on a layer 5 A thick of polysaccharide of pneumococcus type III demonstrates that a number of molecules of antibody are piled up on top of each other and that they must be immobilized there by highly specific forces without coming into contact with the antigenic film. To make sure that there were no large aggregates of antibody present in the antisera used, two of them were analyzed in the ultracentrifuge. They were found perfectly normal, exhibiting the usual albumin and globulin sedimenting boundaries. The calculated constants of sedimentation were $s_0^{20} = 4.0_7 \times 10^{-13}$ (albumin) and $s_0^{20} = 7.0_7 \times 10^{-13}$ (globulin) for one of the sera and $s_0^{20} = 4.0_8 \times 10^{-13}$ (albumin) and $s_0^{20} = 7.3_0 \times 10^{-13}$ (globulin) for the other. The determinations were carried out in the phosphate buffer used for the adsorption experiments.

Screening Action—Numerous data presented in the experimental part indicate that three different types of barriers do not prevent immunological reactions from proceeding between underlying films of antigens and antibodies deposited on top of the screen. The following questions are thus raised: Do the antibody molecules, which are specifically immobilized with a bonding energy strong enough to prevent their washing away with saline solution and water, really rest on top of the screen, or do they in some fashion work their way through the screen? Do the screens have holes, or is the mesh of their fabric wide enough to permit the diffusion of antibody molecules? The available information in regard to surface films would appear to preclude this interpretation.

Films of barium stearate have been studied by electron diffraction by Germer (10) and were shown to consist of closely packed molecules with the hydrocarbon chain perpendicular to the base, in harmony with the optical thickness found for such films. He states that, "The arrangement can be thought of as resembling that of persons of uniform size in a crowded subway car." Even if thermal motion is taken into consideration, it does not seem possible that "thermal holes" would be large enough to permit the passage of a molecule as large as that of a rabbit antibody (molecular

weight 160,000). In extreme cases the molecule would have to force its way through $18 \times 8 = 144$ carbon atoms in order to reach the antigenic layer. If, in the case of Formvar films, the holes (thermal or permanent) were responsible for the reaction, it would mean that the size of the holes and their density would be the same as in stearate or octadecylamine screens, a very improbable coincidence. However, let us assume for the sake of the argument that the "hole" theory is the explanation. Then the barrier should slow down considerably this assumed diffusion process. As a matter of fact, the rate of the reaction is unaffected by the different types of screens. For example, the amount of antibody adsorbed on a screen of Formvar 110 A thick, deposited on three layers of bovine albumin, was 90 A thick, regardless of whether the antiserum was in contact with the slide for 15 or for 40 minutes. Further, the experiments mentioned above on the temperature effect on the reaction lead to a similar conclusion. The decrease in the reaction rate is the same whether there is a screen or not.

Still another series of experiments is difficult to explain by the "hole" interpretation. There has come to our attention at least one system of large reacting protein molecules for which barriers of stearate or Formvar proved very efficient; namely, that of adsorbed molecules of protamine reacting with insulin. This system was investigated by Clowes (7) who found that in the pH range of 5.5 to 7.5 a thick layer of insulin could be adsorbed on a thin layer of protamine. Our experiments were made with the protamine clupein sulfate[3] and showed that a layer of insulin 220 A thick could be adsorbed on a layer of clupein. A screen of one double layer of barium stearate, however, cut down the reaction completely, since the increment observed of 18 A was the same as that found when there was no underlying clupein layer. Experiments with films of Formvar showed that no specific increase took place for screens thicker than 50 A. (There was, however, an increase of 120 and 40 A with screens 17 and 30 A thick, respectively.) There is no reason to believe that a given screen should be more permeable to larger than to smaller molecules. Actually the size of an insulin molecule ($M \simeq 35,000$) is considerably smaller than that of an antibody molecule.

It is commonly assumed that the interaction between insulin and protamine results from Coulomb forces between these 2 molecules oppositely charged in the physiological pH range. From this view it has been further suggested that the efficiency of the stearate screen could be accounted for

[3] A 0.16 per cent solution of clupein sulfate in a 0.015 M veronal buffer, brought to pH 7.2, was smeared on a conditioned (uranyl acetate) stearate gage for 3 minutes. The thickness of the adsorbed layer was 8 to 10 A. The insulin solution was pH 6.5 and contained 18 units per cc. in a 0.05 M veronal buffer. The insulin solution was smeared on the clupein layer or the barrier for 10 minutes.

by a neutralization of the basic groups of clupein by the carboxyl group of stearic acid. This explanation appears scarcely adequate, since in the absence of a screen it would be necessary for the insulin molecules to exchange places with the sulfate ions of clupein in order to react. The screen itself consists of barium stearate which cannot neutralize the already neutralized basic groups of clupein. An exchange reaction might, however, conceivably take place, with elimination of barium sulfate. Whether or not this occurs, the fact remains that the insulin molecules cannot replace the stearate, and this in itself demonstrates the efficiency of the long alkyl chain of the stearate in preventing the charged groups of insulin from approaching the polar groups of protamine.

All the foregoing evidence would appear to be definitely against the "hole" interpretation of the effects observed in the presence of barriers.

TABLE IV

Salt Treatment of Slides Coated with Three Double Layers of Bovine Albumin and Homologous Antibody with and without Intervening Screens

Thickness of Formvar screen on top of 3 double layers of bovine albumin	Thickness of antibody	Thickness of Formvar screen on top of antibody layer	Loss in thickness following salt treatment
A	A	A	A
44	140	0	61
66	99	0	46
0	160	0	63
0	190	0	61
0	195	39	0
0	180	58	0
0	160	118	0

It has been suggested that during the deposition of the screens the antigenic layer or layers work their way up to the top of the screen. This possibility seems hard to conciliate with the following facts. When a slide, after specific adsorption of antibody, is treated with a sodium chloride solution (5 to 10 per cent for 10 minutes), a removal of part of the adsorbed antibody results. In contrast, if a screen of Formvar is deposited on top of the antibody layer, no antibody can be removed with salt treatment, as appears from Table IV which summarizes our findings in this connection. It is obvious that antibody molecules cannot leave the slide when they are covered with a Formvar film or treated with ethylene dichloride. These facts demonstrate that both antigenic film and antibody molecules must stay below the barrier during its deposition. Therefore, if the antibody molecules cannot work their way out of the screen, it also seems highly

improbable that they should be able to work their way down through the screen, a point which adds further evidence against the "hole" theory.

Yet another hypothesis has been advanced; namely, that during the deposition of a barium stearate screen the screen in some way duplicates the distribution of charges of the antigenic film and becomes an artificial antigen. This possibility seems remote, especially when one considers that films of Formvar behave in a fashion similar to that of barium stearate. Experiments in which the screen of Formvar was made on a clean glass slide and then transferred as one unit on the antigenic films, as described above, seem definitely to exclude this hypothesis, since the results were similar to those obtained with screens formed directly on the antigenic layers. The most characteristic property of immunological reactions is their extraordinary specificity. This in itself would seem to preclude the assumption that interaction between antigenic films and antibody, in the presence of a screen, would take place through highly specific induced polarization transmitted through the screen. It was shown earlier in this paper that the influence of uranyl conditioning can be transmitted through many monolayers of protein, presumably by induced polarization, to prevent the top layers from escaping the surface of a slide during the process of deposition of multilayers. In this case, however, the forces involved are entirely non-specific, in contrast to the forces involved in immunological reactions. It would now seem that the only tenable explanation of these immunological phenomena is based on the assumption that long range forces exist between the antigenic films and the antibody molecules, since direct contact between the molecules is unlikely. However, no known chemical force can explain interactions at distances of the order of a few hundred Ångströms. Therefore our knowledge concerning intermolecular forces obtained through the study of relatively small molecules would be inadequate to describe specific interaction between large molecules such as those involved in immunological and enzymatic reactions.

The possibility might then be considered that antigen and antibody interact as two resonating oscillators. A necessary condition for the realization of such long range interaction is that the assumed oscillators are extended; otherwise the interaction would fall down rapidly with the distance. These extended oscillators could be visualized according to the ideas expressed by London (11) which are very pertinent. Extended oscillators are likely to occur in large molecules built on the principle of pattern repetition, like the polypeptide chain of proteins or the glucose-glucuronic acid units of polysaccharide type III. These oscillators would be characteristic of the molecule as a whole, or at least a considerable portion of it. The frequency of these oscillators might presumably be located in the extreme infra-red of the spectrum, possibly in the border

line between optical and electrical spectra. It would then follow that the interaction between antigen and antibody molecules could be interpreted by a field action, rather than by specific chemical reacting groups. From this point of view the idea of valency of antigen and antibody would lose its significance. The specific action would then be explained, though in a more modern language, by a theory related to the early concept of Bordet and his followers.

It is too early to say whether in all immunological reactions the concept of a field action gives a more adequate interpretation of the facts than the idea of reaction between specific chemical groups. At present it can be said that the experiments presented in this article are most readily explained on the assumption of a specific field of force extending over 100 to 200 A. Immunologists have been puzzled for some time by the fact that "antibodies for disaccharide glycosides seem to reflect the pattern of the homologous hapten as a whole" and that "A similar situation was encountered in the study of immune sera to peptides" (12). Such observations would be a natural sequence to the concept of a field action representing the molecule as a whole. It might well be conceived that the nature of the antigen would affect the extension of the oscillators. If the specific resonators were localized in small chemical groups, as in the case of artificial conjugated antigens containing few hapten groups per molecule, then the concept of integrated field action would not be needed and a purely chemical theory perhaps would be adequate. We plan to investigate the screening action of barriers on immunological reactions of artificial conjugated antigens. Some of the observations reported above, besides those dealing directly with screening effects, would definitely be in favor of an integrated field action. If the immunological reaction were of a purely chemical nature, conditioning of one double layer, by blocking certain specific groups, should cut down the subsequent reaction with antibody in the same relative proportion as conditioning of three double layers of bovine albumin. It was shown, however, (see Fig. 2) that uranyl conditioning of a slide covered with one double layer of bovine albumin had little effect on the subsequent reaction with antiserum, whereas conditioning of three double layers reduced considerably the amount of adsorbed antibody. On the other hand, if the large amount of antibody adsorbed on three double layers of bovine albumin results from a cooperative phenomenon between the multilayers of the antigen, it might be expected that the conditions necessary to promote an enhanced field would be the more easily disturbed the greater the number of monolayers in the antigenic pile.

From a biological point of view our findings may lead in the direction towards a rational theory of the dynamics of the living cell. Many puzzling facts confronting the physiologist might conceivably find an

explanation if specific long range forces operate at considerable distances between macro molecules. Limiting ourselves to immunological reactions, we may say that the sphere of influence of antibody molecules in the animal body may extend much further than is commonly believed. Interaction between antigen and antibody could occur through inert layers or a thin cell membrane. In the next article it will be shown that analogous phenomena likewise take place in enzymatic reactions.

SUMMARY

Experiments are described which deal with immunological reactions between films of antigen transferred on metal slides and antibody contained in a drop of solution brought into contact with the slide. The antigens used were egg albumin, bovine albumin, ferritin, and the polysaccharide from pneumococcus type III. The influence of screens deposited on the antigenic films prior to the smearing of the antibody solution on the slide was investigated. It was found that intervening screens of barium stearate, octadecylamine, and Formvar did not altogether prevent a specific immobilization of homologous antibody. The influence of such barriers was mainly a function of their thickness and very little if at all of their nature.

The possibility was considered of holes in the barrier, thus allowing the antibody molecules to come in direct contact with the antigenic film by diffusion. This explanation seems contrary to the experimental evidence. The conclusion is reached that interaction between large molecules, such as those involved in immunological and enzymatic reactions, may take place through a field of specific long range forces extending over 200 A, a distance many times greater than that calculated for the interaction of small molecules.

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THE REACTION OF FORMALDEHYDE WITH PROTEINS*

IV. PARTICIPATION OF INDOLE GROUPS. GRAMICIDIN

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In the study of the reaction of formaldehyde with proteins (2–4) it was noted that some of the aldehyde was bound in a manner not reversible by acid hydrolysis. Wadsworth and Pangborn (5) and Holden and Freeman (6) had previously found that histidine and tryptophane differed from other amino acids in forming stable compounds with formaldehyde. Jacobs and Craig (7) and Neuberger (8) had prepared the formaldehyde derivatives of tryptophane and histidine, respectively, and found that new rings were formed by means of methylene bridges between the amino groups and reactive positions on the indole or imidazole rings. Recently Velluz (9) and Baudouy (10) suggested that the irreversible fixation of formaldehyde by proteins might involve their histidine and tryptophane residues. Similar ideas have been expressed by Nitschmann and Lauener (11) and Swain *et al.* (12).

Gramicidin, the antibiotic isolated by Hotchkiss and Dubos (13) from tyrothricin, and shown to contain no polar groups other than indole and aliphatic hydroxyl groups (14), was found in this Laboratory to react with formaldehyde in neutral or alkaline solution to give a stable derivative of altered biological properties (methylol gramicidin) (15, 16). This, and other observations which will be discussed below, appeared to lend independent support to Baudouy's claim concerning the irreversible fixation of formaldehyde by the tryptophane residues of proteins. However, as was pointed out by Swain *et al.* (12), the mechanism of the reaction could not be assumed to be the same as that for the free amino acid. The present publication is concerned with the conditions favorable for, and the mechanism of the combination of formaldehyde with, tryptophane residues in peptide linkage. The reaction with histidine residues is significantly different and will be discussed separately.

The reaction of gramicidin with formaldehyde served as a model system and was studied in some detail, since gramicidin contains about 40 per cent

tryptophane, 5 times that present in the protein of highest known tryptophane content. Simple indoles were used to substantiate the results with gramicidin. Finally, the conclusions were tested with proteins rich in tryptophane. The data indicate that 3-alkylindole residues react readily in alkaline solution with 1 equivalent of formaldehyde, which adds to the nitrogen or, less likely, the α-carbon atom to give a methylol group as
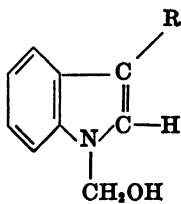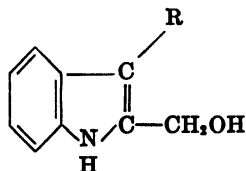
(I)   (II)

indicated in formulas I and II. The reaction is largely reversible in strong alkali but acid liberates only part of the formaldehyde and causes a breakdown of the indole ring.

Methods

Formaldehyde Analysis—The amount of formaldehyde bound by a protein has usually been determined in the following manner (17, 18, 2, 3). The thoroughly washed protein derivative is subjected to a combined acid hydrolysis and distillation, formaldehyde being subsequently determined in the distillate by titrimetric or gravimetric procedures. These conditions, however, do not cause the release of formaldehyde from the methylene compound resulting from its interaction with free tryptophane, and only partial release of formaldehyde bound by the residues of this amino acid in peptide linkage. Analyses for such "stably bound" formaldehyde have been based on determinations of the difference between the amounts added to, and recoverable from, reaction mixtures (12). However, it was found that even this technique could not be applied to tryptophane-containing products, since tryptophane derivatives react with formaldehyde under the conditions of combined acid hydrolysis and distillation (Table I) (11). The same difficulty was encountered when formaldehyde was determined by a chromotropic acid method (19); during the heating in strong sulfuric acid, indoles were found to bind formaldehyde.[1] Thus it was not possible

[1] Besides all indole derivatives, cysteine, dimercaptopropanol, tyrosine, and several proteins containing tyrosine and histidine bound formaldehyde under the conditions of chromotropic acid analysis (30 minutes in 14 N sulfuric acid at 100°) when 0.012 mg of formaldehyde was used in the presence of 0.5 to 3.0 mg. of compound On the other hand, histidine, acetylhistidine, histidine anhydride, and proteins low in tryptophane, tyrosine, and histidine (gelatin, isinglass, protamine) bound no appreciable amounts of formaldehyde under these conditions.

by either technique quantitatively to recover formaldehyde added to indoles after the sulfuric acid. In proteins rich in tryptophane, there appears to be no accurate method available for the determination of formaldehyde bound reversibly, or to determine by difference that bound irreversibly. Baudouy (10) and Nitschmann and Lauener (11) have also emphasized this difficulty. With Baudouy's technique for irreversibly bound formaldehyde actually the sum of that "irreversibly" bound plus that bound in so labile a manner as to be split off during the washing of the protein was determined.

TABLE I

Irreversible Fixation of Formaldehyde by Indole Derivatives and Proteins under Conditions of Analysis

Substance	Formaldehyde bound per mole indole residue
	mole
Indoleacetic acid	0.56
Tryptophane	0.65
Benzoyltryptophane	0.67
Acetyltryptophane	0.59
2,3-Dimethylindole†	0.33
Chymotrypsinogen	0.83
Lysozyme	0.72
Gramicidin‡	0 0

* 5.7 mg. (0.19 mM) formaldehyde were added to 10 to 20 mg. of simple indole derivative or 100 mg. of protein in 50 ml of 1 N sulfuric acid and distilled until fumes filled the distillation flask. Under the same conditions cysteine bound 0.45 equivalent of formaldehyde. Tyrosine, histidine, acylhistidines, and proteins free from, or very low in tryptophane (bovine serum albumin, globin, cattle hoof keratin, gelatin, insulin), bound no significant amounts.

† Distils with steam simultaneously with the formaldehyde

‡ The insolubility and resistance to hydrolysis of gramicidin probably account for its inability to bind formaldehyde under these conditions.

Special methods had therefore to be used in order to determine the extent of combination of formaldehyde with tryptophane in peptide linkage. The peculiar properties of gramicidin made it a most useful experimental material for this purpose. Its insolubility in water permitted its quantitative removal from reaction mixtures which could then be analyzed for residual formaldehyde, with or without distillation. The absence of amino, amide, and guanidyl, the usual polar groups that bind formaldehyde reversibly (2, 3), made it possible to attribute all formaldehyde bound by gramicidin after thorough washing to combination with its numerous tryptophane residues. The amount bound was ascertained (*a*) by differ-

ence between the amount added and that found in the combined supernatant and wash solutions, and (b) by elementary analysis of the derivative.

When simple indole derivatives and acyltryptophanes were used as model substances, the decrease in the formaldehyde content of reaction mixtures was determined directly, without distillation and without separation of the reaction product, by the addition of dimedon and buffer (20, 2, 3). Since the acylamino acid side chain binds no formaldehyde,[2] the total amount bound by all model compounds used could be attributed to combination with the indole group. The data obtained by this technique were at times checked and confirmed by repeated precipitation and resolution of the indole derivatives with water and alcohol and ultimate analysis of the pooled supernatant solutions for residual formaldehyde. Characterization of the modified reaction products or recovery of the unchanged indole derivatives supplied the final proof for the occurrence and the nature of the reaction.

Tryptophane Analysis—All 3-substituted indole derivatives, upon reaction with formaldehyde, lose their ability to give a blue color with Ehrlich's reagent and give instead a purple color of lessened intensity if read with the usual red filter. This color test is similarly altered in formaldehyde-treated proteins and hence supplies a quantitative estimate of the extent of reaction of their tryptophane residues. The analyses were performed according to Horn and Jones (22), either on unhydrolyzed proteins or on enzymatic digests (pancreatin). Alkaline hydrolysis could not be used, since it was found that strong alkali regenerated the chromogenic activity. In addition, alkaline hydrolysis was found to cause some destruction of the tryptophane in chymotrypsinogen (Table IV) and lysozyme. Acid hydrolysis also causes destruction of tryptophane, particularly in the presence of formaldehyde.

Since gramicidin and methylol gramicidin are resistant to enzymatic digestion, these were analyzed either unhydrolyzed, in 50 per cent acetic acid solution, or after acid hydrolysis (16). Acid hydrolysis, even though performed in evacuated Thunberg tubes, led to formation of considerable amounts of humin in the case of methylol gramicidin (about 25 per cent of the nitrogen was rendered insoluble) but not with gramicidin itself, the tryptophane of which is resistant to acid hydrolysis with these precautions.

The Folin method for the determination of combined phenols and indoles was sometimes used as an additional technique. The buffer and reagent, prepared and diluted according to Herriott (23), were added simultaneously

[2] As shown in many control experiments with several acylamino acids in the course of this study. Carpenter (21) and Neuberger (8) have demonstrated the inability of benzoylalanine and benzoylhistidine to bind formaldehyde.

to the appropriately diluted sample and the color was developed during 1 hour at 40°. By digestion of the protein with pancreatin (pH 8 for 3 days at 40°), the quantitative significance of the Folin values could be greatly increased, compared to those obtained on unhydrolyzed proteins. Direct analyses of intact proteins are known to yield values corresponding to only about 60 per cent of those expected; they have been found to be affected by the ease of denaturation of different derivatives (24, 25). In contrast, the chromogenic activity of pancreatic digests of all proteins studied except lysozyme[3] corresponded closely to the sum of their known tyrosine and tryptophane contents. This method of hydrolysis has an additional advantage in that it minimizes the danger of reversal of the modifying reaction.[4]

The formaldehyde derivatives of simple-indoles and of chymotrypsinogen showed lowered chromogenic values with the Folin reagent. This observation, however, did not facilitate the interpretation of the reaction mechanism, since the molecular requirements for a positive test are not known. Alkyl substitution of the indole ring did not greatly affect its chromogenic value with the Folin reagent. All indoles listed in Table IX gave from 50 to 70 per cent of the color of tryptophane, on a molar basis.

Other Analytical Techniques—Hydroxyl groups of gramicidin and methylol gramicidin were determined by quantitative acetylation (29, 14). Resorcinol and cetyl alcohol were 95 and 90 per cent acetylated under the conditions used. The solubility of gramicidin derivatives was estimated spectrophotometrically in a 25 per cent alcoholic medium (16). Nitrogen was determined by the modification of the Kjeldahl-Gunning-Arnold method recently described by White and Secor (30).

Materials—Generous supplies of gramicidin were furnished by the Wallerstein Laboratories. Indole compounds, unless otherwise described, were commercial products. 2,3-Dimethylindole was synthesized by the method of Snyder and Smith (31). 1,2-Dimethylindole-3-acetic acid

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[3] Lysozyme had to be digested with pepsin after heat denaturation in acid solution (26) to release its complete chromogenic value (12.4 per cent, calculated as tryptophane; see foot-note 6).

[4] As an example, this technique has proved useful in establishing the number of phenol sulfate groups formed upon sulfation of proteins with concentrated sulfuric acid (27). An unhydrolyzed sample of insulin that had been sulfated for a short time at −18° appeared to contain 5.7 per cent tyrosine compared to the value of 8.9 per cent found with unhydrolyzed insulin. In enzymatic digests absolute values of 7.7 and 12.2 per cent were obtained, respectively. The latter value agrees with that obtained after acid hydrolysis. The former, when corrected for the amount of sulfate introduced, indicates that 30 per cent of the tyrosine had been sulfated. This product was found to have high hormonal activity (28). In contrast to the sulfate, O-acetyl-tyrosine was not stable under the conditions of enzyme digestion used.

was prepared from the methylphenylhydrazone of ethyl levulinate, as described by Degen (32), except that the condensation was carried out in glacial acetic acid and was catalyzed by boron trifluoride as described

TABLE II

*Rate of Reaction of Gramicidin and Indole Derivatives with Formaldehyde at Room Temperature**

Compound	Final pH†	Moles formaldehyde bound per mole indole residue						
		2.5 hrs.	4 hrs.	6 hrs.	24 hrs.	3 days	7 days	11-12 days
Gramicidin .	11.3	0.8‡	1.0		0.9		1.0	
Skatole	11.3	0.8	0.7	0.9	0.7	0.8	0.8	0.9
"	3.5		0.2		0.4		0 8	
Acetyltryptophane	7§		0.3		0 6	0.8		
Indoleacetic acid	11.3		0.8			0.9	0.9	
" " .	7§		0.3		0.5		1.0	
" "	3.5§						0.7	
2,3-Dimethylindole	11.3				0.5	0.5	0.4	
"	3.5				0.6		0.6	
1,3-Dimethylindole.	11.3				0.1		0 1	
1,2-Dimethylindole-3-acetic acid	11.3				0.0		0.0	

* The reaction conditions were as follows: 1 mM of the indole derivative or 200 to 400 mg. of gramicidin were treated with 5 to 7 mM formaldehyde in a final total volume of 10 ml. Sodium hydroxide, phosphate buffer, pH 8 0, or acetic acid was added to final concentrations of 0.03 M, 0.14 M, or 0.15 M respectively. With the exceptions noted in foot-note § the medium contained 4 to 6 ml. of alcohol At the end of the specified time period, 1 ml. of the reaction mixture was diluted first with 3 ml. of alcohol and then with water to 10 ml. 3 ml. of the diluted solution were added to a mixture of 50 ml of dimedon solution and 75 ml. of acetate buffer (20). After 24 hours, the precipitate was filtered, dried, and weighed. If contamination of the precipitate with the indole reaction product was suspected, nitrogen analyses were run. The results were uniformly negative. With gramicidin, the reaction product was precipitated by the addition of aqueous 0.1 M sodium chloride. After being centrifuged and carefully washed, the product was dissolved in alcohol and again precipitated and washed. The combined supernatant solutions and washings were analyzed for formaldehyde by the dimedon method.

† Measured after dilution with 2 to 3 volumes of water.

‡ That the reaction was practically complete in 2.5 hours was also indicated by the fact that this preparation contained only 20 per cent of the hemolytic activity of gramicidin *in vitro* (15, 16).

§ In aqueous media. Indoleacetic acid was insoluble at pH 3.5 but gradually went into solution during the course of the reaction.

for other indole compounds by Snyder and Smith (31). The properties agreed with those in the literature. Chymotrypsinogen was a commercial preparation that had been recrystallized eight times by E. F. Jansen.

Crystalline lysozyme was kindly furnished by H. L. Fevold and G. Alderton. Tobacco mosaic virus was obtained through the courtesy of W. M. Stanley.

TABLE III

Effect of Reaction Conditions on Properties of Formaldehyde-Treated Gramicidin

Reaction conditions*			Products†			
Formaldehyde concentration	Medium	Temperature	Nitrogen	Tryptophane‡	Hydroxyl groups§ per 10^4 gm.	Solubility‖
per cent		*°C.*	*per cent*	*per cent*		*mg. per cent*
15.0	0.2 M (COOH)$_2$	70	12.7	<5	10	
11.3	0.35 N CH$_3$COOH	70	12.5	<4	6	
2.4	0.15 " "	53	14.2	24	7	0.2
11.3		70	13.7	23		
7.5		53	14.0	<8	10	0.6
2.5		70	13.6	32		
0.4	0.002 N NaOH	53	14.1	<8	11	0.6
7.5	0.02 " "	53	13.6	<8	22	1.8
2.4	0.03 " "	53	13.7	<8	22	2.3
2.4	0.03 " "	Room	13.6	<8	23	2.6
0.5	0 03 " "	53	13.6	<8		
0.1	0.03 " "	53	13.9	16		
15.0	0.08 " "	53	13.5	<8	25	2.3
Control¶			14.5	38	6	0.6

* In most cases, 4 to 20 per cent formaldehyde solution and 1 N acid or 0.1 N alkali were added to 400 mg of gramicidin dissolved in alcohol, in order to give the desired end-concentrations, and the mixture was diluted to 10 ml. The alcohol concentration was 40 to 60 per cent. Reaction time, 2 days at 53° or 70°, 7 days at room temperature.

† Antibacterial activities were as follows the products prepared at 70° and 53° in acetic acid <10 and 73 per cent, respectively; that prepared at neutrality, 93 per cent; and the products of complete reaction in alkali, 81 to 96 per cent of the activity of gramicidin. The hemolytic activities *in vitro* were 10 to 15 per cent for the latter preparations and 50 to 100 per cent for those prepared at 53° at neutrality and in acetic acid. The methods of assay were those described elsewhere (11).

‡ Analyzed without hydrolysis in 50 per cent acetic acid solution; approximate values. An atypical purple color was obtained in all samples designated as containing <8 per cent.

§ By quantitative acetylation (19).

‖ In 25 per cent aqueous alcohol containing 0.125 N sodium chloride (12).

¶ Gramicidin exposed to strong alkali showed slightly lowered N (14.2 per cent) and unchanged tryptophane contents. Treatment with acetic acid at 70° gave a product containing 14.2 per cent N and 34 per cent tryptophane.

DISCUSSION

Extent of Reaction—The data in Table II show that gramicidin in alkaline solution reacts rapidly with formaldehyde, binding almost 1 equivalent

for each tryptophane residue. The reaction proceeds rapidly at room temperature and at low formaldehyde concentrations in the presence of 0.02 N or higher concentrations of alkali (Tables II and III).[5] In neutral solution little formaldehyde appears to be bound. In acid solution the reaction proceeds only at high formaldehyde concentrations and temperatures.

In contrast to gramicidin, acyltryptophanes and simple 3-substituted indoles react at room temperature in acid and neutral as well as in alkaline solution, although at somewhat slower rates (Table II). Like the tryptophane of gramicidin, these model substances bind almost 1 equivalent of formaldehyde.

Tryptophane analyses of formaldehyde-treated chymotrypsinogen, a protein particularly rich in this amino acid[6] (33), demonstrated that the indole groups of a typical protein, like those of gramicidin, react rapidly and even at low formaldehyde concentration in alkaline solution, but not appreciably below pH 8 to 9 (Table IV). Similar results were obtained with tobacco mosaic virus (Table V) and lysozyme, although the insolubility of the alkaline formaldehyde reaction products of these proteins prevented as complete a study as was possible with chymotrypsinogen.[7]

[5] Previously, elevated temperatures (53°) had been used for the gramicidin-formaldehyde reaction (15). The instability of formaldehyde in warm alkaline solution and the possible effect of indoles on its stability in such solutions rendered quantitative work at 53° difficult to control. Main emphasis has therefore been placed in this publication on the more reliable results obtained at room temperature. However, the properties of methylol gramicidin are the same, whether prepared at room temperature or at 53° (in alkaline solution).

[6] Of all proteins studied, lysozyme alone had a higher tryptophane content, that is, 8.0 per cent, corrected for moisture, when analyzed (22) either unhydrolyzed or after enzymatic digestion. After alkaline hydrolysis or acid hydrolysis in evacuated Thunberg tubes, about 4.6 per cent tryptophane was found, compared to 3 8 and 3 3 per cent for chymotrypsinogen under the same conditions. Gramicidin, after acid hydrolysis in Thunberg tubes, appeared to contain 38.1 per cent tryptophane.

[7] The finding of Ross and Stanley (35) that formaldehyde treatment at pH 7 reduced the *Folin color* of unhydrolyzed tobacco mosaic virus was confirmed. However, *tryptophane* analyses (22), both on the intact protein and after enzymatic digestion, indicated that there was no appreciable difference between this derivative and the original protein (2.5 to 2.7 per cent), while the formaldehyde derivate prepared at pH 11 contained less than 1 per cent unchanged tryptophane. It appears that the Folin color of the intact protein, which corresponds to only 53 per cent of the potentially reactive groups, is decreased non-specifically after formaldehyde treatment. Miller (25) found this to be the case for certain acyl derivatives of the virus unless they were previously denatured. Analyses with the Folin reagent after enzymatic digestion confirmed the tryptophane analyses in showing that there was no decrease in chromogenic activity after formaldehyde treatment at pH 7, but a marked decrease after treatment at pH 11. Ross and Stanley's results are thus to be interpreted as reflecting a decreased availability of the tryptophane and tyrosine residues to the Folin reagent after formaldehyde treatment at pH 7.0 rather than

TABLE IV

Reaction of Tryptophane Residues of Chymotrypsinogen with Formaldehyde

	Reaction conditions*			Analytical conditions			
Formaldehyde concentration	Final pH	Buffer		Tryptophane			Phenol + indole‡
			Unhydrolyzed	Enzymatic digest†	Alkaline hydrolysate	Enzymatic digest†
per cent			*per cent*	*per cent*	*per cent*	*per cent*
1	11.5	None	<0.6§	<2.3§	3.3	4.7
1	10.1	Phosphate	<0.7§	<3.1§		
1	9.1	None	4.3	3.8		
1	8.8	Borate‖	4.9	4.2		
1	7.6	None	5 8	5 0		
1	7.5	Phosphate	Insoluble	4.8		
1	6.1	None	"	5 4		
5	11.1	"	<0.6§	<2.6§	3.4	4.6
5	7.4	Phosphate	Insoluble	4.2		
10	10 5	None	<1.0§	<2.0§	3.4	4 7
10	8.2	"	3.9	3.3		
10	7 3	Phosphate	5 0	3 9		
10	6.0	None	Insoluble	3.4		
10	3 8	"	6 1			
10	2 8	"	5 1			
None	11 7	"	6 0	5.3	3.8	9.0
Control			5 8	5 8¶	3.8	10 1

* 50 mg. of chymotrypsinogen were allowed to react in a total of 3 to 4 ml. When buffers were used, these were present in approximately 0.1 M concentration. Otherwise the pH was adjusted with 1 N sodium hydroxide or 1 N hydrochloric acid. After reaction for 1 day at room temperature, the protein was isolated by dialysis and dried by lyophilization. The yields averaged 90 per cent. Rate studies indicated that, at pH 11 to 11 5, the reaction had already proceeded to completion in 1 hour with formaldehyde concentrations of 0.3 or 1.2 per cent.

† Prepared as follows. To 5 to 10 mg. of the protein or protein derivative were added 1 ml of H_2O, 0.1 ml. of 3.4 M phosphate buffer (pH 7.6), and 0.5 ml. of a centrifuged aqueous extract (10 ml.) of 10 mg. of commercial pancreatin. The mixture was held at 40° for 3 days, and then diluted to 10 ml. If solids were still present, they were removed by centrifugation, washed, dried, and weighed. Aliquots of the soluble fraction were subjected to colorimetric, and generally also Kjeldahl, analyses.

‡ Calculated as tryptophane. Without hydrolysis, the values were 3.2 and 3.6 per cent, after alkaline hydrolysis 8.7 and 9.6 per cent, respectively, for treated and control preparations.

§ Based on readings of an atypical purple color.

‖ Under conditions patterned after a technique of toxoid formation (34), that is, upon treatment with 0.25 per cent formaldehyde in borate buffer, pH 8 to 9, for 2 weeks at 40°, the apparent tryptophane content of chymotrypsinogen was reduced to 2.0 per cent.

¶ When formaldehyde was added to the digest (1.2 per cent of the protein), only 4.2 per cent tryptophane was found.

Mode of Combination of Formaldehyde—Earlier investigators (7) have shown that the stable reaction product of tryptophane with formaldehyde is a cyclic compound (2,3,4,5-tetrahydrocarboline-4-carboxylic acid), the formaldehyde creating a methylene bridge between the amino group and the α-carbon atom of the indole nucleus.   It remained to be determined whether formaldehyde acted on tryptophane in peptide linkage in a similar manner, yielding acyltetrahydrocarbolinecarboxylic acid derivatives, or whether the aldehyde was bound in some other manner, the most likely being an addition, as a methylol (hydroxymethyl) group, to the indole

TABLE V

Reaction of Tryptophane Residues of Tobacco Mosaic Virus with Formaldehyde

Reaction conditions*			Analytical ‖				
			Phenol + indole†		Tryptophane		
Formalde-hyde concen-tration	Buffer	Final pH	Unhydrolyzed	Enzymatic digest‡	Unhydrolyzed	Enzymatic digest‡	Alkaline hydrolysis
per cent			*per cent*	*per cent*	*per cent*	*per cent*	*per cent*
2	Phosphate	7.0	0.65	6.3	2.7	2.6	2.7
1.4	None	11.0	Insoluble	4.6	Insoluble	<1.0§	2.4
Control			3.5	6.6	2.5	2.6	3.1

* The experiments at neutrality were patterned according to Ross and Stanley (35): 3 per cent protein in 0.1 M, pH 7, phosphate buffer. To obtain the alkaline medium 0.5 ml. of N NaOH was added to 3 5 ml. of this virus solution, then water (1.5 ml.) to give a final protein concentration of 1.9 per cent. Reaction was permitted to proceed for 20 hours at room temperature.

† Calculated as tyrosine.

‡ Both untreated tobacco mosaic virus and that treated with formaldehyde at pH 7.0 had to be heat-denatured before enzyme digestion could proceed to such an extent that there was maximum release of Folin chromogenic activity. This was not necessary for chymotrypsinogen, serum albumin, and insulin. With regard to lysozyme, see foot-note 3.

§ Based on an atypical purple color.

nucleus.   The properties of the formaldehyde derivatives of gramicidin clearly indicate the formation of methylol groups.   Thus, elementary analyses agree with the methylol but not with the methylene formulation (Table VI).   Further, quantitative acetylation (29) indicates the appearance of about one new hydroxyl group for each equivalent of formaldehyde combining with each tryptophane residue (Table III).   The number of hydroxyl groups introduced can consequently be used as an additional

indicating actual combination of these residues with formaldehyde.   The observation of Ross and Stanley that simple indoles react with formaldehyde at pH 7 is confirmed in the present study.

means of ascertaining the rate of, and the conditions favoring, the gramicidin-formaldehyde reaction (Table III). The introduction of methylol groups into gramicidin is in line with the increased water solubility of the formaldehyde derivative (Table III) (16).

Further confirmatory evidence for the methylol configuration is derived from the comparative lability of the linkage. Treatment of methylol gramicidin with hot aqueous sulfuric acid, either under the conditions of hydrolysis and distillation used to determine reversibly bound formaldehyde or of the chromotropic acid method (19), liberated one-fifth to one-third of the formaldehyde bound by gramicidin. That the liberated formaldehyde was not merely adsorbed was indicated by the constancy of

TABLE VI

*Composition of Gramicidin and Methylol Gramicidin**

	C	H	N	Hydroxyl groups per 10^4 gm.†
	per cent	*per cent*	*per cent*	
Gramicidin‡	62.8	7.44	14.5	6
Gramicidin-formaldehyde product	61.2	7.40	13.6	25
Calculated for methylol " §	61.5	7.40	13.7	24
" " methylene " §	63.4	7.62	14.1	6

* Averages of repeated analyses on many preparations, on a dry basis.

† By quantitative acetylation (29). Model substances gave values of 90 to 95 per cent of the theoretical.

‡ From the known amino acid composition of gramicidin, Synge (36) calculates that 144 C atoms should be present for 30 N atoms. The data of Hotchkiss and Dubos (13), Tishler (37), and the analyses recorded here indicate 148, 153, and 152 C atoms for each 30 N atoms, respectively.

§ Based upon the assumption that gramicidin contains 40 per cent tryptophane (14, 16, 36) (19.6 residues per 10^4 gm.) and that each typtophane residue adds one methylol (or methylene) group.

this value for many different preparations, including both extensively heated and repeatedly recrystallized samples (1). Reactive phenolic methylol compounds, such as 2,6-dimethylol *p*-cresol, are known to release some formaldehyde under similar conditions (38). Model experiments showed that under the distillation conditions used with gramicidin methylol skatole and methylol 2,3-dimethylindole released 0.20 and 0.53 equivalents of formaldehyde, respectively.   ·

More striking is the effect of sodium hydroxide on methylol gramicidin. This agent seems to split off the methylol group quantitatively. Thus, alkali-treated methylol gramicidin shows the full chromogenic value of the original gramicidin. Its hydroxyl groups are reduced to the number

occurring in the original gramicidin, and it corresponds in elementary analyses closely to a control sample of gramicidin treated with alkali and isolated in the same manner (Table VII). Skatole and 2,3-dimethyl-indole could be regenerated from their respective methylol derivatives under the same conditions. The chromogenic activity of other simple indoles, abolished by reaction with formaldehyde, is also largely restored

TABLE VII

Effect of Alkali on Methylol Gramicidin

Conditions of treatments*			Analytical results			
Sodium hydroxide concentration		Temperature	Tryptophane content		N	Hydroxyl groups per 10^4 gm §
			Unhydrolyzed†	Acid-hydrolyzed‡		
	N	°C.	per cent	per cent	per cent	
Methylol gramicidin	None		<10¶	(<7.5)¶**	13 6	25
	5	78	37–47	34.5		
	5	40††	35–45	36.2	14.2‡‡	6
	5	23	27			
	1	23	<14¶			
	0.1	23	<8¶			
Gramicidin	None		35–45	38.1	14.5	6
	5	40		34.1	14 2‡‡	

* In aqueous suspension. Reaction period, 1 day. When high temperatures were used, insoluble products were obtained.

† Approximate values. The curve for unhydrolyzed gramicidin does not coincide with the standard curve for tryptophane

‡ By a mixture of glacial acetic acid and 6 N hydrochloric acid (0 8:3) 18 hours at 100°.

§ Calculated on the basis of the original gramicidin

¶ Based on the atypical purple color.

** Insoluble humin (containing 25 per cent of the total nitrogen) separated during hydrolysis.

†† This preparation contained 55 per cent of the hemolytic activity of gramicidin, compared to the value of 10 to 20 per cent found with numerous preparations of methylol gramicidin.

‡‡ These alkali-treated preparations of gramicidin and methylol gramicidin also had identical carbon (61.4, 61.4 per cent) and hydrogen (7.3, 7.3 per cent) contents.

by the action of sodium hydroxide (Table VIII). It is not surprising that 1,3-dimethylindole forms an exception, since this compound was found to yield a 2-methylene rather than a methylol derivative.

The tryptophane in formaldehyde-treated proteins was found to resemble that of methylol gramicidin in regaining much of its chromogenic value upon treatment with sodium hydroxide (Tables IV and V). This may be

taken as evidence that protein indole groups, like those of gramicidin, add the formaldehyde as methylol groups, an assumption which could not as readily be proved by analytical means for proteins as it could be for gramicidin.[8]

Location of Methylol Groups—The location of the methylol groups on the indole nucleus remains to be discussed. As previously mentioned, 3-substituted indoles lose, upon reaction with formaldehyde, their chromogenic activity with the p-dimethylaminobenzaldehyde reagent. The same is the case with 1,3-dimethylindole (Table VIII). These observations appeared to provide evidence favoring addition of the formaldehyde to

TABLE VIII

Chromogenic Values of Indoles and Formaldehyde Derivatives by Horn-Jones (22) Method for Tryptophane*

Compound	Molar chromogenic value	Comments
	per cent	
Skatole .... .. ı	78	Blue-gray, fades
Methylol skatole†	0	Brownish
Benzoyltryptophane	94	Blue, fades
" formaldehyde-treated (pH 3 5)	34	Purple
Indoleacetic acid	88	Blue, fades
" " formaldehyde-treated (pH 7)	17	Purple
1,3 Dimethylindole	57	
Methylene bis(1,3-dimethylindole)†	0	Faint red

* 2,3-Dimethylindole and carbazole (2,3-phenyleneindole) gave no color at the usual time of reading and a slow development of a bluish color later.

† Treatment with 5 N sodium hydroxide at 53° for 18 hours regenerated chromogenic activity from methylol skatole but not from methylene bis(1,3-dimethylindole) (*cf.* Table VII).

the 2-carbon atom, since all indoles with free 2 positions, and only these, give this typical intense blue color.[9]

Further evidence for the location of the methylol groups was sought on a more strictly chemical basis than was afforded by colorimetry. The fact

[8] At elevated temperatures (70°), formaldehyde caused marked losses in the tryptophane chromogenic activities of proteins even at neutrality, apparently more readily than is the case for gramicidin under such conditions This has been tentatively attributed to the formation of methylene bridges between aminomethylol groups and indole rings. This reaction will be discussed in a subsequent publication in conjunction with similar reactions involving other cyclic amino acid residues.

[9] However, a slow development of a purple-blue color was observed with carbazole and 2,3-dimethylindole.

that skatole and indoleacetic acid and its homologues bind up to 1 equivalent of formaldehyde agrees with the combination of the aldehyde with either the nitrogen or the α-carbon atom, without favoring either position (Tables II and IX). It serves to exclude the side chain of tryptophane from playing a rôle in the reaction. The properties of our methylol skatole, however, differ significantly from those ascribed by Plant and Tomlinson (39) to 2-methylol skatole. Particularly, the lability of the skatole-formaldehyde reaction product in alkali differentiates it from the known hydroxymethylindoles and favors its formulation as an N-methylol compound.

TABLE IX

*Reaction of Simple Indole Derivatives with Formaldehyde**

Compound	Final pH	Formaldehyde bound per mole compound
		mole
Benzoyltryptophane	6.8	0.9
Indole-3-propionic acid	6.8	0.9
Indole-3-acetic acid	6.8	1.0
Skatole	11.1	1.0
	3.5	0.6
2,3-Dimethylindole	11.1	0 5
	3.5	0.7
1,3-Dimethylindole	11.1	0 1
	3 5	0 4†
1,2-Dimethylindole-3-acetic acid	11.1	0 1

* 1 mM of compound was allowed to react with 4 to 8 mM of formaldehyde in 10 ml. total volume for 2 days at 53°. The alkaline reaction mixtures contained 0.03 N sodium hydroxide; acid mixtures, 0 1 N acetic acid.

† The reaction product crystallized and was identified as methylene bis(1,3-dimethylindole) Thus, the reaction had proceeded to about 80 per cent completion.

A comparison of the reactivity of 1,3- and 2,3-dimethylindoles towards formaldehyde should demonstrate unequivocally the mode of attachment of the methylol group. The results obtained with these model indoles were, however, not quite conclusive (Tables II and IX). Both at room temperature and at 53°, 2,3-dimethylindole bound about 0.5 equivalent of formaldehyde in alkaline and acid media. The reaction product was separated from unchanged 2,3-dimethylindole and found to correspond to methylol dimethylindole in its composition. In contrast, 1,3-dimethylindole did not react appreciably in alkaline solution; in acid solution a crystalline product which separated proved to be methylene bis(1,3-dimethylindole).[10] (1,2-Dimethylindole-3-acetic acid bound no appreci-

[10] A small amount of a crystalline product (less than 10 per cent) could also be isolated from alkaline reaction mixtures. Its nitrogen content (9.2 per cent) cor-

able amounts of formaldehyde in either alkaline or acid solution.) Thus, it appears that, in alkaline solution, only 2,3-dimethylindole combines with formaldehyde, although the reaction does not proceed as readily and completely as it does with indoles substituted only in the 3 position. The properties of methylol skatole and the behavior of the dimethylindoles thus favor the attachment of the formaldehyde to the nitrogen, while the colorimetric evidence pointed towards an involvement of the 2 position of the indole nucleus. By analogy with the behavior of phenols and with other indole reactions, one may assume that the formaldehyde primarily reacts with the nitrogen but then may rapidly migrate to the 2 position if this position is unsubstituted. However, it is equally possible that the migration of the methylol group to position 2 occurs only during the colorimetric test, which is always performed in hydrochloric acid. This possibility is supported by the observation that both methylol gramicidin and methylol skatole undergo immediate reactions when treated with hydrochloric acid at room temperature or at 0° in glacial acetic acid solution. Both methylol products are transformed into yellow to red-brown precipitates, while unmodified gramicidin and skatole are unaffected by similar treatment. The nitrogen content of neither methylol compound is altered, but their chromogenic activities can no longer be regenerated by treatment with alkali. Thus, while it has not been possible to crystallize the acid-treated methylol skatole or to identifiy it with Plant and Tomlinson's (39) 2-methylol skatole, indications are that the acid conditions of the test cause the change responsible for the loss in chromogenic activity of formaldehyde-treated indoles. Thus, the evidence favors the attachment of the methylol group to the nitrogen as indicated by formula I.[11]

Of two other attempted approaches to the problem, one has given no answer and the other favored the above conclusion. Acetic anhydride treatment of gramicidin in glacial acetic acid (in contrast to pyridine) has been found to yield a product containing considerably more acetyl groups than correspond to its hydroxyl content, which thus have been allocated to the only other polar groups, that is, the indole groups (16). Since this product had the full chromogenic value of gramicidin and gave off acetic

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responded to methylene bis(1,3-dimethylindole); yet it differed from this product, as formed in acid media, by a slightly higher melting point (158°). The mixed melting point between the two showed a marked depression (125–130°).

[11] Other gramicidin derivatives carrying substituents on the indole nitrogen, notably the sulfamate and the acylated products, show the chromogenic value corresponding to their original tryptophane contents (16). The different behavior of these and the formaldehyde derivative is understandable in view of the known reversibility, by acid hydrolysis, of all but the formaldehyde fixation. Thus the sulfate group or various acyl groups tend to be split off rather than to migrate to the 2 position.

acid upon acid hydrolysis, it was regarded as a 1-acetyl derivative.[11] It was hoped that formaldehyde treatment of this material might throw light on the mechanism of the formaldehyde reaction.   However, no conclusions could be drawn from the observed ability of this derivative to react with formaldehyde, since the acetyl groups were found to be largely split off during the reaction with formaldehyde in alkaline solution.

On the other hand, acetylation (in glacial acetic acid) of methylol gramicidin introduced acetyl groups corresponding in number to the hydroxyl groups (25 per 10^4 gm.), but introduced no additional amount such as might have been expected if acetylation of the indole nitrogen had occurred (16).   This may be regarded as additional evidence that the methylol groups are located in the 1 position.

Preparative

Methylol Skatole—To 786 mg. (6 mM) of skatole, dissolved in 40 ml. of ethanol, were added 2.25 ml. of N sodium hydroxide and 30 ml. of 3.85 per cent aqueous formaldehyde.   After 6 days at room temperature the mixture was diluted with much water, extracted twice with ether, and the ether washed with water until no more formaldehyde could be detected in the washings.   The ether was dried and evaporated.   The residue (857 mg., crystals and oil) was extracted with cold petroleum ether, then dissolved in benzene, and petroleum ether added.   Crystals separated from both solutions (360 and 260 mg.), melting point 52° after recrystallization from benzene and petroleum ether.   In contrast to skatole, this compound gave no color with tryptophane reagents (Table VIII).

$C_{10}H_{11}ON$.   Calculated.   C 74.5, H 6.9, N 8.7
                 Found.   " 74.5, " 7 0, " 8.7

When 55 mg. of methylol skatole were suspended in 1 ml. of 5 N sodium hydroxide and heated to 53° for 2 hours, the crystals turned into an oil, then crystallized.   The product was identified as skatole by melting point and mixed melting point.

Methylene Bis(1,3-dimethylindole)—A solution of 1 ml. of 1,3-dimethylindole in 7.5 ml. of alcohol was mixed with 2 ml. of 38.5 per cent aqueous formaldehyde, and 0.5 ml. of 3 M acetic acid and held at room temperature for 7 days.   The deep red solution deposited crystals which were isolated after cooling, and washed with cold alcohol.   Yield, 540 mg.   Recrystallization from alcohol yielded colorless crystals, m. p. 138–148°.

$C_{21}H_{22}N_2$ Calculated.   C 83.5, H 7.3, N 9.3, mol. wt. 302
               Found   " 83 2, " 7.3, " 9 2, "   " (Rast) 327, 314

The same compound but in somewhat smaller yield (303 mg.) was obtained after a similar mixture had been heated at 78° for 2 hours.

Methylol 2,3-Dimethylindole—725 mg. (5 mM) of 2,3-dimethylindole were treated with formaldehyde as was skatole. After 4 days at room temperature, a 1 ml. sample was diluted for formaldehyde analysis (which indicated that 0.49 equivalent had been bound by the indole). The rest of the solution was worked up as above. From the residue (743 mg.), unchanged starting material was extracted with boiling petroleum ether (b. p. 30–60°). The residue from this operation (340 mg.), after recrystallization from benzene-petroleum ether, melted at 98–100°.

$C_{11}H_{13}ON$ Calculated. C 75.5, H 7 4, N 8.0
                Found.   " 75 5, " 7 5, " 7 9

Treatment of 38 mg. of methylol 2,3-dimethylindole with 5 N sodium hydroxide at 53° for 20 hours, yielded 29 mg. of 2,3-dimethylindole, characterized by melting point, mixed melting point, and nitrogen analysis (9.7 per cent).

Comments

The present investigation has demonstrated that formaldehyde reacts with gramicidin only on the tryptophane residues. The fact that the toxicity is thereby decreased, whereas the antibacterial activity is retained (15, 16), suggests that tryptophane residues in other biologically active compounds may also play an important rôle in mediating their activities. For example, the mode of detoxication of toxins with formaldehyde to form toxoids has often been the subject of speculation. The work of Farrell (34), as confirmed by Dubos and Geiger (40), with Shiga toxin suggests that the reaction of formaldehyde may be that of combining with part of the indole residues. This worker found that incubation of the crude toxin with 0.5 per cent formaldehyde failed to detoxify the material even after 18 months at 37°. By adjustment of pH, he observed a reduction of toxicity to one-seventh at pH 6.7 and to one-fiftieth at pH 8.2, without loss of the antigenic activity. The maximum loss of toxicity and retention of antigenicity were observed at pH 8.5 after 2 weeks at 37°. Under similar conditions, the tryptophane residues of chymotrypsinogen reacted to about 67 per cent (Table IV, ‖ foot-note). Although various other polar groups react with formaldehyde under such conditions, none of these reactions is known to be as dependent upon an alkaline pH as is that of the tryptophane residues. Thus, a reaction mechanism analogous to that of gramicidin may be the basis of the formation of some formalin toxoids, as was suggested by Velluz (9).

Any generalizations, however, would appear to be premature in view of the effectiveness of ketene (41) and of acetic anhydride in pyridine (42) for the preparation of some toxoids and vaccines. These acetylating agents

and conditions may not affect the indole groups of gramicidin,[12] nor have they been shown to act on these groups in any typical protein.

Thus, there are indications that the desirable effects (reduction of toxicity without loss of antigenicity) may be produced by modifying the indole groups in some and the amino, or possibly both types of groups, in other toxic proteins. However, the tobacco mosaic virus protein, probably the most exhaustively studied in this regard, represents further problems, inasmuch as it is not inactivated by acylation of its amino groups (43), but is inactivated by formaldehyde (35, 44) under conditions in which few if any of its indole groups appear to react.

It is of interest that "hydroxyalkylation" has been shown to reduce the toxicity of several pharmacologically active synthetic compounds (45).

SUMMARY

Gramicidin binds rapidly in alkaline solution an amount of formaldehyde equivalent to its tryptophane content. In acid solution the reaction proceeds only at high temperature and formaldehyde concentrations.

The formaldehyde adds as methylol groups probably to the nitrogen of the indole rings. Formaldehyde is released only partially during acid hydrolysis, but completely and without damage to the gramicidin molecule in strong alkali.

The chromogenic activity of the tryptophane residues is largely abolished through combination with formaldehyde and regenerated by strong alkali.

The tryptophane residues of proteins appear to react with formaldehyde under the same conditions and in a manner similar to those of gramicidin At room temperature there is little reaction at pH 7 to 8, even at high formaldehyde concentration, but at pH 11 the reaction is completed rapidly, even at low formaldehyde concentration.

All 3-substituted indole derivatives, including proteins rich in tryptophane, bind formaldehyde in boiling N to 20 N sulfuric acid.

Simple 3-substituted indoles bind up to 1 mole of formaldehyde at room temperature, both in alkaline and in acid solution. 2,3-Dimethylindole reacts incompletely, even at elevated temperature. 1,3-Dimethylindole reacts appreciably only in acid yielding methylene bis(1,3-dimethylindole). Pure methylol derivatives were obtained from skatole and 2,3-di-

[12] Experiments to introduce acetyl groups on the indole residues of gramicidin with ketene were ambiguous. A vigorous stream of ketene was passed into a suspension of gramicidin in a 1 M solution of sodium acetate for 30 minutes (at 0°). The solution was kept slightly alkaline by the simultaneous addition of 1 M sodium hydroxide. The isolated gramicidin was found to have an acetyl content corresponding only to its hydroxyl groups (six per 10^4 gm.) but not to its indole content (19.6 per 10^4 gm.). Somewhat more acetyl was introduced when gramicidin was extensively ketenized in alcoholic solution.

methylindole. 1,2-Dimethylindole-3-acetic acid does not react with form-aldehyde.

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NOTES ON THE USE OF REDISTILLED WATER AND OF AMYLOSE IN THE ESTIMATION OF SERUM IODINE

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In the original description of the permanganate acid ashing micromethod for iodine determinations (1), the technique was given for redistillation of water from a reservoir containing potassium carbonate or hydroxide. No data were presented to emphasize the importance of making the water alkaline and of redistillation. When tap water, which frequently contains more than 10 γ of iodine per liter, is distilled in equipment of the type customarily employed in chemical laboratories, the product may contain more than 0.5 γ of iodine per liter. This is as much as that present in 10 cc. of serum from a euthyroid individual. A commercial "demineralizer" yielded water free of iodine for only a short time. On the other hand, when distilled water was made alkaline and redistilled in a glass flask, the product was regularly found to contain no determinable iodine in a 500 cc. sample.

In the permanganate ashing method as previously described (1), the color in the visual titration may vary from pink to purple. When the "soluble" starch was replaced by amylose (2), the color was a clear blue, but the titers were found to vary appreciably with the concentration of amylose.[1] For example, 0.05 cc. of 0.01 per cent amylose gave theoretical titers, but with 0.05 cc. of 0.025 per cent amylose, high titers were found; complete color did not develop originally in proportion to the micro amounts of iodine; photometric zero points of amylose-iodine solutions changed within 5 minutes or less in solutions containing salts and large amounts of sulfuric acid.

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[1] Amylose in the form of a 20 per cent paste in butyl alcohol, obtainable from the G. Frederick Smith Chemical Company, was employed.

DINICOTINYLORNITHINE: A METABOLITE OF NICOTINAMIDE IN THE °CHICKEN

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In the course of experiments on the effect of high levels of nicotinamide in the diet of the chick (1) an attempt was made to identify the form or forms in which the nicotinamide was excreted. Early attempts to identify the metabolites excreted by birds ingesting nicotinic acid had been made by Komori and Sendju (4), who gave daily doses of 1 gm. of nicotinic acid in the form of the sodium salt for 10 days both to a pigeon and to a hen. The urine was collected and from the pigeon 3.5 gm. and from the hen 3.7 gm. of nicotinic acid were recovered; the authors wrote "Aus der von Nikotin-säure abfiltrierten Lösung konnten wir eine zweite Substanz nicht auffinden." The experiments described below show, however, that a characteristic derivative of nicotinic acid is excreted by the chicken.[1]

Estimations of the nicotinic acid content of the droppings of chicks fed a mash containing 2 per cent added nicotinamide were made by us before and after hydrolysis with 2 N alkali. It was found that the apparent nicotinic acid content after hydrolysis was 20 to 40 per cent higher than before, indicating the presence of a metabolite containing nicotinic acid and developing in the König reaction less color than the nicotinic acid which it contained. Application of the method of Sarett (6) failed to show the presence of any methylated derivatives of nicotinic acid such as trigonelline or N-methylnicotinamide in the excreta.

When fresh droppings were boiled for an hour in 5 times their weight of water and centrifuged, the unchanged nicotinic acid plus metabolites could be concentrated by adsorption on Lloyd's reagent at pH 0.6, and subsequent elution with 0.5 N KOH. Such elutes, on adjustment to pH 1 or 2, were extracted continuously with ethyl ether, which removed unchanged nicotinic acid. The extracted aqueous solutions then had an apparent nicotinic acid content after alkaline hydrolysis 4.5 times greater than before, with the König reaction with CNBr and metöl. Of known

[1] A preliminary account of this work has been published (2).

nicotinic acid derivatives nicotinuric acid shows the greatest ratio of apparent nicotinic acid contents after and before hydrolysis, its ratio being 4.1. These measurements on partly purified extracts of chick droppings therefore indicate the presence of a previously unknown metabolite.

Isolation of Metabolite

Since the development of this work depended upon the use of a simple technique both for the identification and the quantitative estimation of the metabolite during the course of the isolation, a description of this technique is given first.

Colorimetric Determination of Metabolite—The unknown substance can be measured quantitatively by the König reaction for pyridine derivatives employing CNBr and aniline, according to the following procedure. To 5 ml. of an aqueous solution of the metabolite there are added 5 ml. of ethanol (95 per cent), 1 ml. of 1 M phosphate buffer (pH 7.0), 4 drops of saturated aqueous aniline, and 0.4 ml. of 3 per cent CNBr solution. After standing 20 minutes the yellow color is measured in the Evelyn colorimeter against a reagent blank prepared in the same manner, with the 420 mμ filter. A solution containing 15 γ of nicotinic acid, treated as above, was used as the reference standard. Treatment of the metabolite with CNBr and aniline as described, following hydrolysis for 1 hour in 2 N KOH, resulted in a 6-fold increase in the color produced. The ratio of 1:6 of the color intensity before and after alkaline hydrolysis was used as an index to identify the unknown substance qualitatively. This method of employing aniline instead of metol was used in place of the method of Perlzweig, Levy, and Sarett (5), on account of its greater speed.

Isolation of Metabolite from Excreta—Droppings were collected over a period of 6 days from 3 to 4 week-old chicks maintained on a practical ration containing an added 2 per cent nicotinamide. After drying in an oven at about 125° for 24 hours, the excreta were ground to a fine powder, and weighed 640 gm. The dried material was extracted by boiling for 1 hour with three 1 liter portions of absolute methanol. The alcohol was removed from the extract by vacuum distillation and the residue taken up in 200 ml. of water.

This dark colored extract, which contained the metabolite as well as large amounts of unchanged nicotinic acid, nicotinamide, and other impurities, was adjusted to pH 3.5 with dilute H_2SO_4 and placed in a continuous ether extractor for 48 hours. The ether extract at the end of this time contained all of the free nicotinic acid and nicotinamide present in the original extract, as well as a portion of the dark pigments. There was no loss of the metabolite by this process.

The dark aqueous solution, now free of nicotinic acid, was warmed on

the steam bath to free it from ether and then cleared of pigments by stirring for 1 hour with 25 gm. of basic lead acetate. The filtrate after removal of excess lead with H_2SO_4 was evaporated to about 100 ml. on the steam bath under a current of air. This solution was adjusted to pH 10 with Ba(OH)$_2$, distilled under vacuum until free of ammonia, and the solution was finally evaporated to near dryness. The last traces of water were removed by vacuum desiccation.

The dried material, which is extremely hygroscopic, was extracted by stirring for 30 minutes with three 100 ml. portions of absolute ethanol at 50°. The residue from this treatment contained insignificant amounts of the metabolite and was discarded. The alcoholic filtrates and washings were evaporated to about 150 ml. and placed in the ice box overnight. The dirty gray precipitate which formed was removed, washed with cold ethanol, and discarded. The alcoholic solution was evaporated almost to dryness, the residue taken up in 150 ml. of H_2O, and the barium removed quantitatively with H_2SO_4. The $BaSO_4$ precipitate was washed free from adhering amounts of the metabolite by boiling with water. The filtrate and washings were evaporated on the steam bath under a current of air to a thick amber-colored oil.

The oil was taken up in 75 ml. of 95 per cent ethanol; 70 ml. of saturated alcoholic HgCl$_2$ solution were added and finally the solution was saturated with powdered HgCl$_2$. The mixture was allowed to stand overnight at room temperature. The voluminous precipitate (43 gm.) was removed by filtration and boiled with three 50 ml. portions of 95 per cent ethanol. These alcoholic washings were discarded. The residue was then boiled with successive portions of water until the water washings no longer became cloudy on cooling. These washings were also discarded. There remained after this treatment 13 gm. of water-insoluble, amorphous HgCl$_2$ salt of the metabolite and of other substances.

The amorphous material was ground to a fine powder, suspended in 75 ml. of water, and the mercury removed with H_2S. After filtering and washing the H_2S precipitate with hot water, the filtrate and washings were evaporated almost to dryness. The dark oily residue was taken up in 50 ml. of 95 per cent ethanol and treated with a saturated alcoholic solution of picrolonic acid. After standing for 2 hours, the solution was concentrated to one-half of its volume, cooled, and filtered with suction. The very dark filtrate which contained no significant amount of the metabolite was discarded. There were obtained from this treatment 4.0 gm. of a picrolonate which was recrystallized from boiling water. The picrolonate of the metabolite at this point was contaminated with a small amount of the picrolonate of at least one other base, which could not be separated by fractional crystallization. The mixed picrolonates were dissolved in

75 ml. of hot dilute hydrochloric acid, cooled, and the picrolonic acid removed by extraction with ether. The aqueous acid solution was evaporated to a dark, viscous residue, which was very hygroscopic and could not be crystallized. This dark residue was dissolved in 10 ml. of water, and titrated potentiometrically to the first inflection point (pH 5.5 to 7.0) with 1 N KOH solution. The water was removed by evaporation and the · residue dried in a vacuum desiccator. The dried residue (still hygroscopic) was extracted by boiling with three 5 ml. portions of absolute ethanol; the extracts were cooled; the precipitate (KCl) was removed and discarded; the filtrate was evaporated and finally dried in a vacuum desiccator. The dried dark hygroscopic residue was stirred for a few minutes with 5 ml. of cold (15°) absolute ethanol and filtered with suction. After washing with cold ethanol there remained on the filter 700 mg. of the metabolite as a white non-hygroscopic solid.

Structure of Metabolite

Properties of Metabolite—The metabolite was obtained as its potassium salt, a white crystalline solid melting at 262–263° (in a copper block previously heated to 236°) after two recrystallizations from 98 per cent ethanol. It is soluble in water and alcohol, sparingly soluble in absolute ethanol, and insoluble in ether, acetone, and chloroform. A small portion, when burned down on a platinum lid, gave a white ash, which was alkaline in reaction. The compound is of basic nature, as is evidenced by the formation of insoluble derivatives with $HgCl_2$, $AuCl_3$, picric acid, and picrolonic acid.

An elementary analysis supports an empirical formula of $C_{17}H_{17}O_4N_4K$, corresponding to a molecular weight of 380.4.

Calculated.   C 53.67, H 4.50, N 14.73, K 10.28
Found.   " 53.93 " 4.42 " 14.40 " 10.50
                    " 53.64 " 4.19 " 14.76 " 10.28

The nicotinic acid content of the compound was observed to be 62.8 and 63.2 per cent in duplicate analyses; theoretical value is 64.7.

Heating an aqueous solution of the compound with CNBr at 75° for 5 minutes, cooling, and adding saturated aqueous aniline gave the characteristic yellow color produced by pyridine and its derivatives. This indicates the presence of pyridine nitrogen in its uncombined tertiary state. The substance was also treated with CNBr and metol by the method of Perlzweig *et al.* (5) for the colorimetric determination of nicotinic acid, and shown to give a color equivalent to 0.16 of that produced by nicotinic acid. The results are recorded in Table I. A similar measurement carried out after boiling the substance for 1 hour with 2 N KOH showed a 4.04-fold increase in the color, indicating the liberation of a nicotinic acid-like

substance during hydrolysis. Calculated as nicotinic acid, this substance represented 63.0 per cent of the compound. The metabolite is not hydrolyzed by boiling with 6 N HCl for 1 hour.

Treatment of a small amount of the metabolite with ninhydrin gave no color reaction. However, the addition of this reagent to a neutralized alkaline hydrolysate of the metabolite produced an intense violet color. This indicates the liberation of an amine or an α-amino acid during the hydrolysis. The extreme stability of the compound to acid hydrolysis would perhaps indicate a peptide-like substance rather than an amide. A large sample of the metabolite was hydrolyzed with alkali and the components isolated and identified as nicotinic acid and ornithine in the following manner.

TABLE I

Comparison of L Values Obtained by Colorimetric Determination of Metabolite before and after Hydrolysis, with Two Different Amines in Color Reactions

To bring the readings within the accurate range of the instrument, 100 γ of dinicotinylornithine were used for the readings in the first line, but only 10 γ for those of the second line, for which the greater color of the products of hydrolysis was measured.

	L value *	
	Metol method (Perlzweig et al (5))	Aniline method, present paper
100 γ dinicotinylornithine	0.421	0.124
10 γ after alkaline hydrolysis	0.170	0.075
7 19 γ nicotinic acid†	0.171	0.074
10 γ nicotinic acid	0.238	0.105

* Evelyn photoelectric colorimeter.
† The amount of nicotinic acid obtained by hydrolysis of 10 γ of dinicotinylornithine.

Hydrolysis of Metabolite—To 850 mg. of the metabolite were added 25 ml. of water and 9 gm. of $Ba(OH)_2 \cdot 8H_2O$, and the mixture was boiled under reflux for 3 hours in the absence of CO_2. There were added 0.7 ml. of 10 N NaOH solution and CO_2 was passed through the still hot solution until the precipitation of barium was complete. The precipitated $BaCO_3$ was washed by boiling four times with 15 ml. portions of water. The filtrate and washings were adjusted to about pH 3 with HCl, the CO_2 allowed to boil off, and the solution was evaporated to a volume of about 50 ml.

Isolation and Identification of Nicotinic Acid—The hydrolysate (pH 3), which contained 570 mg. of the nicotinic acid-like substance expressed as nicotinic acid, was placed in a continuous ether extractor for 24 hours. At the end of this time the ether contained by direct measurement with

CNBr and aniline all the nicotinic acid-like substance originally present in the hydrolysate. The aqueous solution (now free of the nicotinic acid-like substance) was reserved for the isolation of the amine-like substance. The ether extract was evaporated to dryness; the slightly discolored residue was taken up in 30 ml. of water and cleared by shaking with about 200 mg. of Darco charcoal. The solution was evaporated to 20 ml. and placed in the refrigerator for 2 days. The crop of crystals was washed with cold water, alcohol, and finally ether. The yield was 425 mg. of small needle-like crystals. The melting point was 237–238° (uncorrected). The mixed melting point with nicotinic acid was unchanged. A sample of the isolated material gave the same amount of color when treated with CNBr and metol, according to the method of Perlzweig et al. (5), as did an equal weight of nicotinic acid. Nitrogen determinations by the method of Shirley and Becker (8) gave identical values with the isolated substance and with nicotinic acid. It is thus established that the nicotinic acid-like substance produced by the hydrolysis of the metabolite with alkali is identical with nicotinic acid.

Isolation and Identification of Ornithine—The aqueous hydrolysate now freed of nicotinic acid by ether extraction was evaporated to a volume of about 10 ml., adjusted to pH 9.7 with KOH, and the evaporation continued nearly to dryness. The residue was extracted by boiling with three 5 ml. portions of absolute ethanol and the remaining residue (KCl) was discarded. After cooling in an ice bath, the alcoholic extract was filtered and the ethanol removed from the filtrate by evaporation. The residue was taken up in 10 ml. of H$_2$O, adjusted to pH 7.0 with HCl, and the slightly discolored solution cleared by boiling for a few minutes with 100 mg. of Darco charcoal. After filtering, the clear colorless filtrate was taken to dryness. The white residue was refluxed with 3 ml. of 75 per cent ethanol until dissolved. Absolute ethanol was added until a faint cloudiness appeared. The solution was placed in the refrigerator until crystallization was complete. There were obtained, after filtering and washing with absolute alcohol, 225 mg. of a white crystalline compound as its hydrochloride. The substance melted at 210–211° (uncorrected) after two recrystallizations from 80 per cent ethanol.

A chloride determination showed the compound to contain 21.1 per cent chlorine, indicating a molecular weight of 168. The melting point, the molecular weight, and the fact that chicks have been shown to conjugate administered benzoic acid with ornithine suggested the possible identity of the above substance. The mixed melting point of the isolated substance and the *dl*-ornithine hydrochloride was 210–211° (uncorrected). Ornithuric acid was prepared from *dl*-ornithine, according to the method of Schulze and Winterstein (7). A similar derivative prepared from the

isolated material also melted at 181–182°. The mixed melting point with ornithuric acid was unchanged. It is thus established that the amine-like substance produced by the hydrolysis of the metabolite with alkali is identical with ornithine.

On the basis of the foregoing evidence the metabolite contains ornithine and nicotinic acid coupled by a peptide link. Nicotinic acid and the amino acid are present in the molecule in the molar ratio of 2:1, as indicated by the elementary analyses and by the content of nicotinic acid. The metabolite therefore has the structure shown in the accompanying diagram.

$$CH_2\text{---}CH_2\text{---}CH_2\text{---}CH\text{---}COOH$$

(2,5-Dinicotinylornithine)
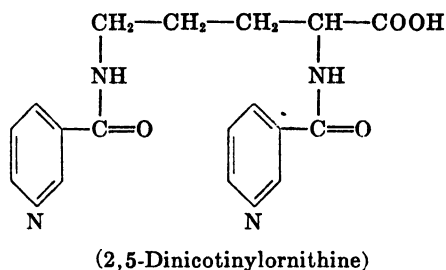
It is of interest to note that this formula is exactly analogous to that of ornithuric acid (dibenzoylornithine), which is excreted by the chicken after feeding benzoic acid (3).

SUMMARY

The excreta of chicks appeared to contain more nicotinic acid after alkaline hydrolysis than before, indicating the presence of a hydrolyzable compound of nicotinic acid.

This substance has been isolated from dried chick droppings. A useful step in the isolation was the removal of uncombined nicotinic acid from a crude extract containing both free and combined nicotinic acid by continuous extraction of the aqueous solution with ethyl ether at a low pH.

The metabolite containing combined nicotinic acid was identified as dinicotinylornithine. After hydrolysis the nicotinic acid and ornithine were isolated and identified.

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THE URINARY EXCRETION OF A
FAT-MOBILIZING AGENT*

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The work of Schoenheimer and his collaborators (1) has amply demonstrated that the apparent constancy of the quantity of fat in the normal adult mammal results, not from the chemical inertia of the fat depots, but rather from the nice balance between processes leading to the deposition of fat and processes leading to its mobilization and utilization. A decrease in the magnitude of the fat depots may occur incident to diminution in the rate of fat synthesis, and indeed such a retardation has been shown to occur in thiamine deficiency (2) and in diabetes (3). The magnitude of the fat depots may obviously also decrease as a result of increased mobilization of depot fat, and the fact that lipemia and fatty liver are frequently observed during starvation or uncontrolled diabetes suggests that this process is contributing to the loss of adipose tissue.

An agent of biological origin endowed with the specific capacity of accelerating the mobilization of depot fat has been described by Best and Campbell (4) as occurring in the anterior pituitary gland. This pituitary adipokinin[1] which may be concentrated from aqueous extracts of beef anterior pituitary glands by precipitation, at pH 5.2, with 2 volumes of alcohol, produces, when injected into the fasted mouse, an increase in the quantity of liver fat which is, within limits, linearly related to the dose injected. Thus the analysis of the mouse liver for fat provides a convenient assay method for this adipokinetic principle (5). That the fat which accumulates in the liver after the administration of pituitary adipokinin arises at the expense of preexisting depot fat has been shown by Barrett, Best, and Ridout (6), and again by Stetten and Salcedo (7). The physiological significance of this depot fat-mobilizing material is indicated by the finding that hypophysectomized rats, kept on a suboptimal diet, lost less depot fat than did control animals on the same diet (8); conversely, forced feeding of hypophysectomized rats led to excessive adiposity (9–11).

The possibility that the rate of mobilization of depot-fat is influenced

[1] To attain brevity, we propose the word "adipokinin" to describe any material which favors the mobilization of depot fat and the adjective "adipokinetic" to describe this activity.

by the liberation, from the anterior pituitary gland, of a specific adipokinetic substance, has therefore been investigated. In these experiments whole pituitary glands were removed from freshly killed normal adult rats. One, two, or three such glands were finely ground with 1 cc. of saline, and the resultant pulp injected subcutaneously into an adult female mouse. After 10 hours without food, the mouse was killed and the liver was pulverized with an excess of anhydrous Na_2SO_4. This powder was extracted for 48 hours with $CHCl_3$ in a Soxhlet apparatus, and the weight of the fat-soluble material determined after removal of the solvent. In Table I the effect upon mouse liver fat of the injection of one, two, or three rat pituitaries is compared with a control series in which 1 cc. of saline was injected.

TABLE I

Effect of Injection of Whole Rat Pituitary Glands upon Fat Content of Livers of Fasting Mice

The whole pituitary glands from one, two, or three normal adult rats were finely ground in 1 cc. of saline and the entire mass injected into a fasting mouse. The fat content of the mouse liver was determined after 10 hours.

No. of rat pituitaries per mouse	No. of mice	Weight of mice	Mean liver weight	Mean liver fat	Mean liver fat per 100 gm. body weight
		gm.	*gm.*	*mg.*	*mg.*
0	21	20–26	1.466	74.3	328 ± 13*
1	9	20–23	1.535	138.8	628 ± 34
2	10	20–26	1.771	211.6	937 ± 41
3	5	21–25	1.757	303.4	1167 ± 78

* Probable error $= \pm 0.6745 \sqrt{\dfrac{\Sigma\ (v^2)}{n(n-1)}}$.

The proportionality between the quantity of pituitary material injected and the quantity of accumulated liver fat, previously reported by others (5), is at once apparent. This approach was therefore applied to a study of the problem of variations in the adipokinetic activity of pituitary glands that might result from fasting or from diabetes. From a few preliminary experiments, however, it was apparent that no significant variations from the normal would be demonstrable by this crude assay technique when the pituitary glands of previously fasted or alloxan-diabetic rats were compared with normal glands.

The possibility was therefore entertained that, in spite of the absence of demonstrable variation in the adipokinetic activity of the pituitary glands, in these conditions, variations might occur in the quantity of adipokinin released under conditions of starvation or diabetes, and that this might in turn be reflected by variations in the analogous activity of

the urine.   24 hour urine samples were collected from pairs of young rabbits of opposite sex.   The pH of the urine was adjusted to 5.2 by the addition of HCl, and 2 volumes of alcohol were added.   The resultant dried precipitate was treated with 2 cc. of very dilute Na_2CO_3 solution, centrifuged, and 0.6 cc. of the clear supernatant injected into a mouse.   After 10 hours of fasting, the mouse livers were analyzed for total lipide, as described above.   The same procedure was repeatedly performed on the same rabbits with one variation.   Prior to and during certain of the periods of urine collection, the rabbits were fed *ad libitum*, while other periods of urine collection were preceded by 24 hours of fasting, which was continued during the period of collection.

From the values in Table II, it will be seen that the livers of mice receiving urine extract from normally nourished rabbits are no richer in fat

TABLE II

Effect of Fasting upon Adipokinetic Activity of Rabbit Urine

An extract from the urine of pairs of rabbits has been assayed by the injection into fasting mice and determination of the fat content of the mouse liver after 10 hours.   Urine samples from normally fed, from previously fasted, and from diabetic rabbits have been compared with saline by this procedure.

Material injected	No. of mice	Weight of mice	Mean liver weight	Mean liver fat	Mean liver fat per 100 gm. body weight
		gm.	*gm.*	*mg.*	*mg.*
Saline control ...	21	20–26	1.466	74.3	328 ± 13
Extract, normally fed rabbit urine	15	20–27	1.422	76.0	339 ± 9
" fasted rabbit urine	15	20–26	1.530	124.5	513 ± 20
" diabetic rabbit urine	7	22–25	1.487	80.1	382 ± 14

than the livers of the saline-injected controls.   In other words, the urine of normally fed rabbits was devoid of demonstrable adipokinetic activity. When the same rabbits were fasted, however, there appeared in the urine a material which was precipitated at pH 5.2 by 2 volumes of alcohol and which provoked a statistically significant increase in the fat content of mouse livers.   It is at this time a matter of speculation whether the adipokinetic activity that we have demonstrated to occur in the urine of the fasted rabbit is identical with that shown by Best and Campbell (4) to occur in the normal anterior pituitary gland.   It is of interest, however, that, during fasting, under conditions in which an increase in the mobilization of depot fat might be anticipated, there appears in the urine a material capable of provoking such an increase, which at least superficially resembles pituitary adipokinin.

A few rabbits, rendered diabetic by the intravenous injection of 150

mg. of alloxan monohydrate per kilo, have similarly been studied. When these animals were fed *ad libitum*, their urines contained at most scanty adipokinetic activity, in the small series studied probably not statistically significant. When assays were attempted on the urines of fasted diabetic rabbits, the urine extracts proved uniformly fatal to mice, and the analyses could therefore not be completed.

SUMMARY

A material capable of provoking an increase in the fat content of the mouse liver appears in the urine of the fasted, but not of the normally fed rabbit. Like the fat-mobilizing substance of the anterior pituitary gland, this material is precipitable at pH 5.2 by 2 volumes of ethanol, and its possible relationship to pituitary adipokinin has been discussed.

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A MICROMETHOD FOR THE QUANTITATIVE DETER-
MINATION OF THE URINARY COPROPORPHYRIN
ISOMERS (I AND III)*

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Previous communications (1, 2) have dealt with the problem of determin-
ing the relative percentage of the naturally occurring urinary copropor-
phyrin isomers, types I and III. In the first of these papers a method was
described for separating small amounts of the pure isomers by means of
their differential elution from aluminum oxide with 30 per cent acetone.
In spite of a trial of many alternative procedures it has not been possible to
apply this method consistently to urine except when the coproporphyrin is
first isolated as the crystalline ester. This of course defeats the primary
objective of the method, since the coproporphyrin I ester crystallizes much
more quantitatively than does the coproporphyrin III ester, and relatively
more of the latter is therefore lost in the mother liquor. In addition, a
tedious extraction procedure is required to purify the porphyrin from a
large volume of urine. The second paper referred to described a difference
in fluorescence stability of the two porphyrins which permits determination
of their relative percentage in a given urine sample, without recourse to
isolation. It employs as little as 100 cc. of urine, or even less in instances in
which the porphyrin concentration is considerable. The purpose of the
present communication is to describe details of a method based upon this
difference in behavior.

Method

The concentration of the total urinary coproporphyrin is first determined.
The procedure is based upon similar methods previously described by Saillet
(3), Fischer (4), and Fikentscher and Franke (5).

100 cc. of urine are acidified with 10 cc. of glacial acetic acid and then
extracted three times with 0.5 to 1 volume of peroxide-free ethyl ether, each
time. The combined ethers are washed three times with 3 per cent aqueous
sodium acetate solution. The coproporphyrin is then extracted by four

shakings with 10 cc. portions of 5 per cent HCl. The combined 5 per cent HCl solution is made acid to Congo red paper by addition of saturated aqueous sodium acetate solution, after which the porphyrin is transferred to ethyl ether by means of three extractions. The combined ether is washed twice with water. The porphyrin is then removed from the ether by four extractions with 1 per cent HCl solution, sufficient in amount to
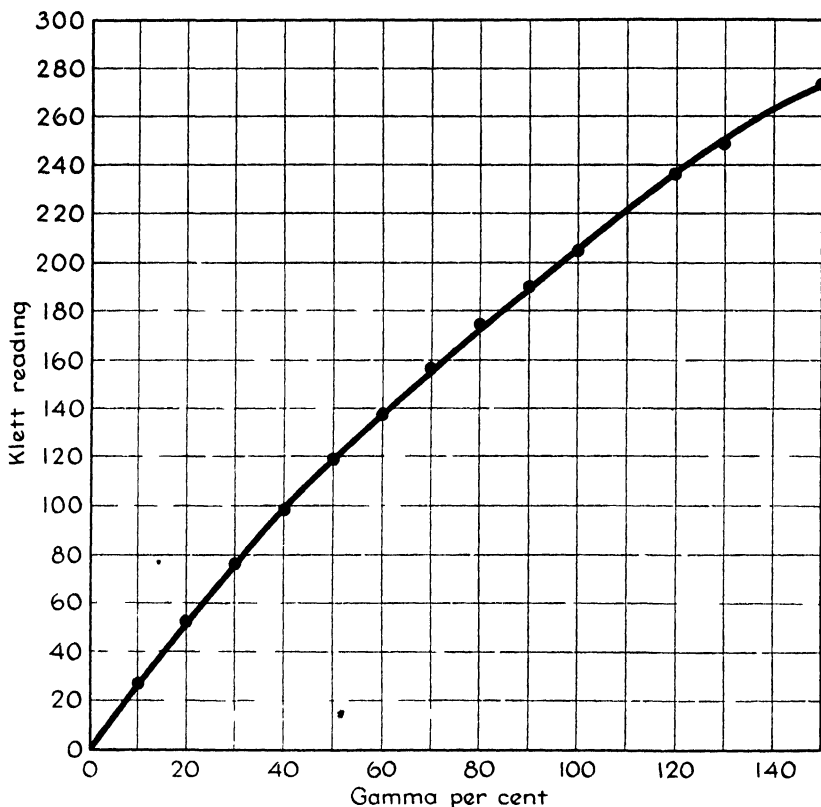
FIG. 1. Fluorescence intensity of varying concentrations of free coproporphyrin in 1 per cent HCl as measured on a Klett fluorophotometer. A 3 per cent aqueous fluorescein solution was employed as the standard.

make a total volume of 10 to 20 cc. Quantitative extraction is insured by observing the red fluorescence of the porphyrin in ultraviolet light.[1]

The total coproporphyrin content of this solution is measured fluorimetrically. The calibration curve obtained with our Klett fluorophotometer is shown in Fig. 1. When the total amount of porphyrin in the final 1

[1] Portable black light, model ZB-11 (Magna Flux Corporation, Chicago, Illinois).

per cent HCl is less than 10 γ, it is best to extract more urine in the same way before proceeding with the isomer analysis, although the latter can be accomplished with as little as 2 γ of total porphyrin. This may be necessary, since considerable loss of porphyrin may occur during the subsequent purification procedure.

Two alternative procedures have been employed for the further purification of the total coproporphyrin and for the isomer analysis. Both of these will be described here, though the isomer analysis by the more recently developed second method is undoubtedly more reliable, and is now used exclusively in our laboratory. The first procedure (A), if carefully executed, has also proved satisfactory. It has been used in many of our earlier clinical studies, to be reported in separate communications. The second of these methods (Procedure B), on the other hand, has been used exclusively in a series of studies to be reported in the "Plutonium project record" (6).

Procedure A—The combined 1 per cent HCl solution is added to 4 volumes of methyl alcohol previously saturated in the cold with HCl gas. After standing at least 4 hours, the porphyrin ester is extracted by chloroform, the methyl alcohol-HCl solution first having been diluted with several volumes of water. The chloroform is then washed successively, twice each with 10 to 20 cc. portions of water, 10 per cent ammonium hydroxide, and 7 per cent sodium chloride. It is then filtered through a chloroform-moistened filter paper and concentrated to dryness on a water bath.

The porphyrin ester is next purified by chromatographic analysis. The residue is dissolved in a drop or two of chloroform and diluted with 3 to 4 cc. of benzene. This solution is passed through a column of calcium carbonate (Cenco, U. S. P., precipitated powder), 6 to 8 cm. in height in a Zechmeister-Cholnoky chromatograph tube (7). The porphyrin esters are adsorbed together in the upper few mm. of the column. The chromatogram is developed with a 1:10 to 1:15 mixture of chloroform-benzene, the higher concentration of chloroform being required in some instances for sufficiently rapid development. The porphyrin zone, containing both coproporphyrin isomers, is eluted to about the middle of the column. The column is then pushed out of the tube and the porphyrin zone is cut out in the usual way (7–9). If at least 5 γ of porphyrin are present, a distinct red or pink zone may be seen in visible light. In ultraviolet light, however, the red fluorescence permits the porphyrin zone to be more sharply outlined with much smaller amounts.

The porphyrin esters are eluted from the CaCO₃ with a small amount of chloroform. This is filtered through a small sintered glass filter. The total porphyrin concentration in this solution is measured in the fluorophotometer. The calibration curve prepared with our Klett apparatus is

shown in Fig. 2. An aliquot containing 4 to 10 γ is removed to an Evelyn tube and evaporated to dryness. The residue is dissolved in 4.5 cc. of freshly distilled acetone, and 10.5 cc. of distilled water are added with immediate mixing. The tube is then corked and the fluorescence is measured immediately in the Klett fluorophotometer.[2] The solution is then
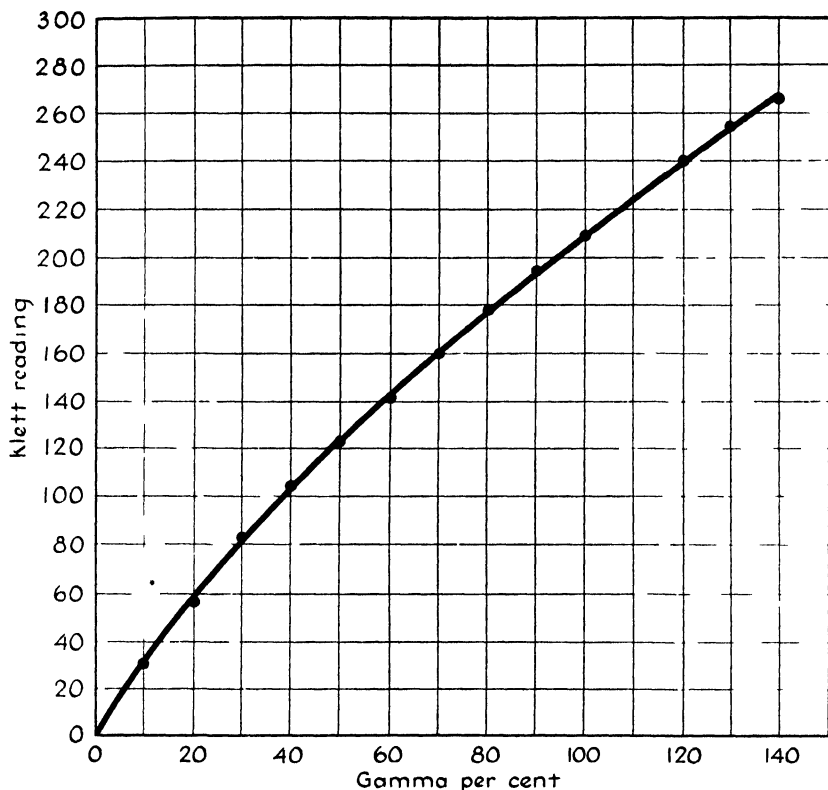
FIG 2. Fluorescence intensity of coproporphyrin methyl ester in chloroform as measured on a Klett fluorophotometer against a 3 per cent fluorescein solution

refrigerated at 4° for 1½ hours, after which it is removed to room temperature for 5 minutes. The fluorescence is then measured again.

As has been shown elsewhere (2), the fluorescence of coproporphyrin I is completely quenched in this interval, whereas that of coproporphyrin III remains essentially constant. In a mixture of the two isomers, however, coprecipitation of both occurs (6). Under the conditions of the test the

[2] A special metal adapter was constructed which permits the use of Evelyn tubes rather than the cuvettes or small tubes supplied with the instrument.

extent of this coprecipitation is such that the 1½ hour reading approximates that of the coproporphyrin III alone.   This is shown by the data in Fig. 3.
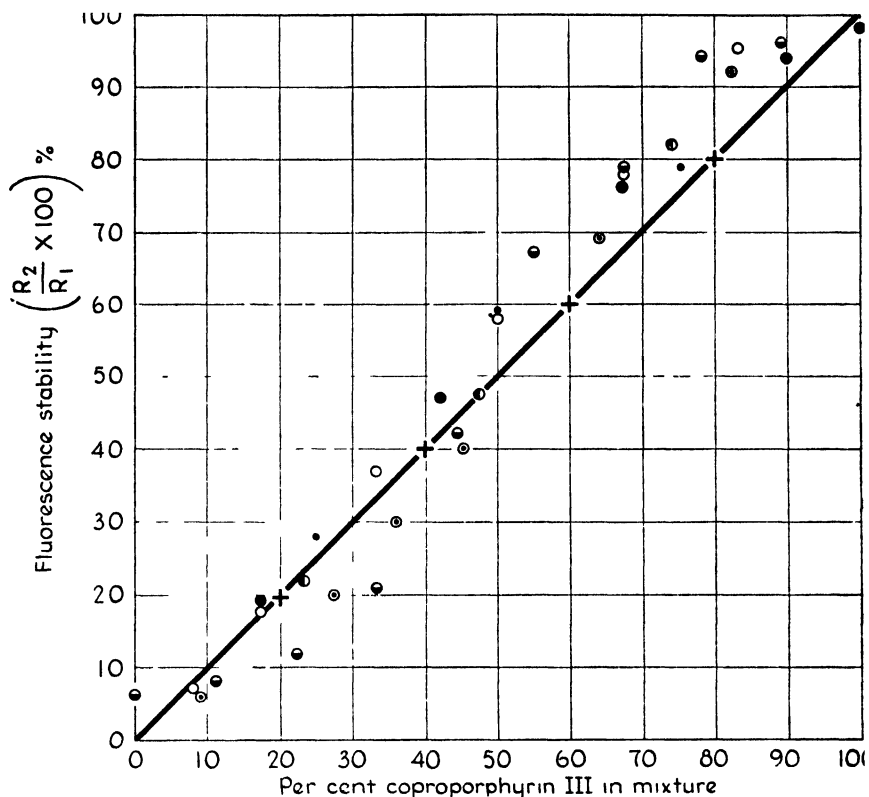
FIG. 3. The relationship of fluorescence stability to the per cent distribution of coproporphyrin I and III esters in 30 per cent acetone (Procedure A). A value of 100 per cent stability indicates no quenching of fluorescence under the conditions of the test.

The per cent of each isomer in the mixture is, therefore, calculated as follows: initial reading = coproporphyrin I + coproporphyrin III; final reading = coproporphyrin III; difference = coproporphyrin I.   Therefore

$$\frac{\text{Final reading} \times 100}{\text{Initial reading}} = \% \text{ coproporphyrin III}$$

It should be emphasized that the reading at 1½ hours does not represent a condition of equilibrium, and that accurate timing is therefore of crucial importance.   It is because of this limitation that isomer analysis by this method has now been replaced by the modified Procedure B which does

yield a condition of near equilibrium for 24 hours or more. It should also be pointed out that the curve shown in Fig. 3 appears to be an S-shaped skew rather than a straight line.

Procedure B—The combined 1 per cent HCl solution is made acid to Congo red paper by the addition of saturated aqueous sodium acetate solution. A few cc. of glacial acetic acid are added and the porphyrin is extracted twice with ethyl acetate. It is then removed from the ethyl acetate by three extractions with 1 cc. portions of 10 per cent HCl. To this are added 10 volumes of $CH_3OH-H_2SO_4$ (20:1). After 4 to 24 hours 3 volumes of 2 per cent NaOH are added and the porphyrin ester is immediately extracted with about 50 cc. of ethyl acetate. The solution should be definitely alkaline; extraction is incomplete if more than a small amount of sulfuric acid remains. The ethyl acetate is washed once with 0.5 per cent NaOH solution, and twice each with water and 7 per cent NaCl. It is then filtered through paper moistened with ethyl acetate and evaporated to dryness on a water bath. The residue is dissolved in chloroform-benzene and subjected to chromatographic purification, as described above for Procedure A.

The concentration of the total coproporphyrin ester is determined in the chloroform eluate of the esters from the $CaCO_3$. An aliquot containing 2 to 5 γ is transferred to an Evelyn tube and evaporated to dryness for isomer determination. This residue is dissolved in 3.5 cc. of freshly distilled acetone, and 6.5 cc. of H_2O are added with constant mixing. The tube is tightly corked, and the fluorescence is measured immediately in the Klett fluorophotometer. The tube is then immersed for 3 or more minutes in a dry ice-acetone bath. The solution is quickly frozen solid. The sample is then removed and kept at room temperature (22–25°). The fluorescence is measured again after a period of from 4 to 36 hours, during which time a condition of relative equilibrium prevails (6).

The ratio of initial to final readings has been determined in a series of 75 experiments in which known mixtures have been analyzed in this way (6). The results are summarized in Fig. 4, in which each point represents the average of five to eleven determinations.

Determination of the per cent of each isomer in any unknown mixture is determined by reference to Fig. 4. Thus, for example, the calculated values for the 75 recovery experiments mentioned above differed by an average of only 2.2 per cent from the known values.

Recovery Data and Confirmatory Isolations

Determination of Coproporphyrin Isomers I and III Added to Extracts of Human Urine, by Procedure A—The first 5 per cent HCl extract from a sample of human urine was found to contain 60 γ per 100 cc. of total

coproporphyrin, of which 20 per cent was type III isomer and 80 per cent was type I. To five portions of the extract each measuring 7.5 cc., copro-
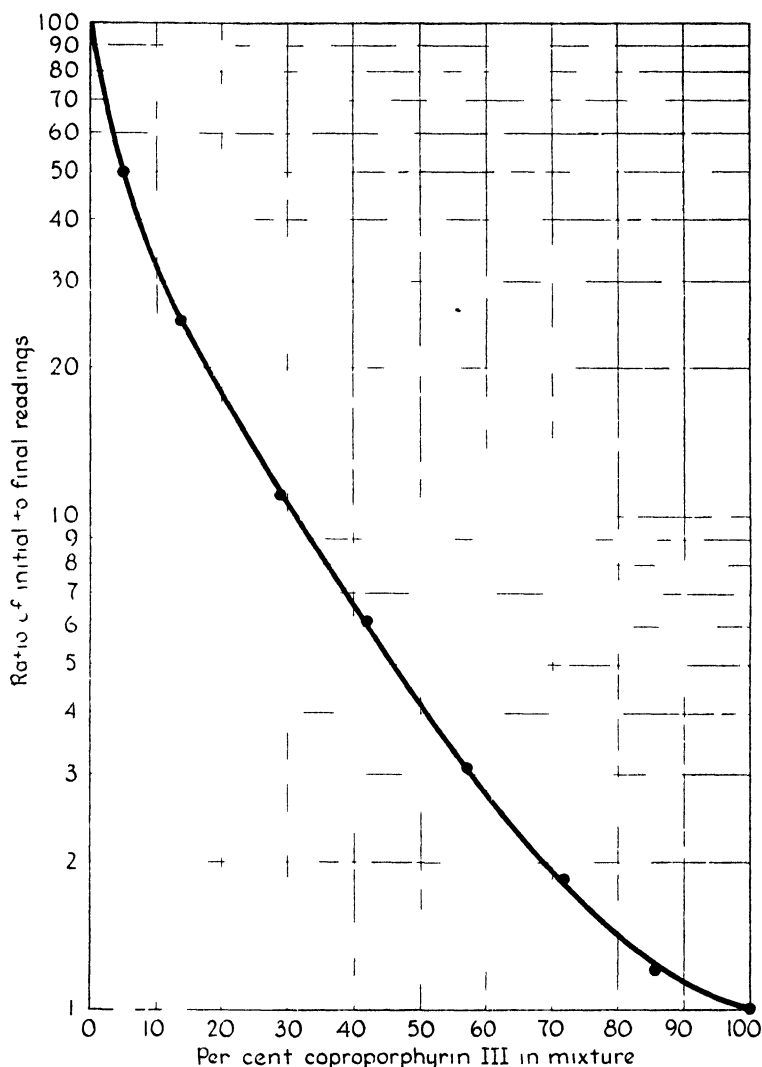
FIG 4 The relationship of the ratio of initial to final fluorescence intensities and the per cent of coproporphyrin III in a 35 per cent acetone solution of a mixture of the coproporphyrin isomers (Procedure B)

porphyrin isomers were added in the amounts noted in Table I, with recoveries as indicated

Comparison of Isomer Analysis by Procedure A and Melting Points of Crystalline Esters Isolated from Larger Volumes of Same Urine—The data of Table II are shown simply to indicate the reliability of the quantitative procedure as controlled by actual isolation of the crystalline coproporphyrin esters from the urine, with determination of the ester melting points.

TABLE I

Recovery of Coproporphyrin Isomers from Urine by Procedure A

Type I added	Type III added	Type III	
		Observed	Calculated
γ	γ	*per cent*	*per cent*
10.5	0.0	5	6
7.5	3.0	23	26
3.9	6.6	50	50
1.5	9.0	61	66
0.0	10.5	75	76

TABLE II

Comparison of Isomer Analysis and Crystalline Ester Melting Points (after Three Crystallizations from $CHCl_3$-CH_3OH)

Urine sample No.	Isomer analysis		
	Coproporphyrin I*	Coproporphyrin III†	M.p.
	per cent	*per cent*	*°C.*
1	98	2	249–250
2	84	16	228
3	79	21	232
4	75	25	228
5	40	60	198
6	25	75	132–134
7	20	80	138
8	18	82	140–141
9	18	82	130
10	11	89	132

* Ester m.p. 252°.

† Ester m.p. 134°, 142°, 170° (dimorphic m.p.).

The method of isolation was the same as that described above except that larger volumes of urine were used. The chloroform eluate from the $CaCO_3$ was evaporated to about 1 cc., about 5 cc. of CH_3OH were added, and the solution evaporated to about 1 cc. Crystallization occurred on cooling. The melting points were determined by means of a Fisher-Johns micro melting point apparatus. It is well known that mixtures of the coropor-

phyrin esters (I and III) exhibit melting points intermediate between those of the pure substances, rather than a sharp depression (9–11). Furthermore, as noted above, the melting point cannot be used as a

TABLE III

Determination by Procedure B of Coproporphyrin I and III Added Directly to Human Urine

Sample No.	Porphyrin added		Coproporphyrin III at 21 hrs.	
	Coproporphyrin I	Coproporphyrin III	Theoretical	Found
	γ per cent	γ per cent	per cent	per cent
1. A	0.0	0.0	86	87
B	0.0	0.0	86	84
C	0.0	0.0	86	86
D	0.0	0.0	86	88
2. A	3.5	0.0	55	50
C	3.5	0.0	55	51
D	3.5	0.0	55	55
3. A	8.8	0.0	38	39
C	8.8	0.0	38	31
D	8.8	0.0	38	39
4. A	17.6	0.0	24	23
B	17.6	0.0	24	28
C	17.6	0.0	24	26
D	17.6	0.0	24	24
5. B	0.0	8.8	93	86
C	0.0	8.8	93	86
D	0.0	8.8	93	89

TABLE IV

Independent Isomer Analyses by Procedure B

Sample No.	Total coproporphyrin per 24 hr. sample	Per cent type III isomer found by two analysts	
		V. H.	D. S.
	γ		
1	175	45	42
2	226	63	64
3	145	34	32
4	452	12	13
5	25	17	23

quantitative measure of the ratio of the two isomers, but merely indicates the predominant isomer type.

Isomer Analyses by Procedure B after Addition of Varying Amounts of Type I and III Isomers Directly to Human Urine—Varying amounts of

free coproporphyrin in 1 per cent HCl were added directly to 500 cc. portions of a pooled sample of human urine which contained 7.1 γ of total coproporphyrin per 100 cc. Just before esterification the final 1 per cent HCl extracts were divided into four equal portions, Samples A, B, C, and D. Samples A and B were esterified by means of CH₃OH-HCl, as in Procedure A, while Samples C and D were esterified with CH₃OH-H₂SO₄. Isomers in all four portions were determined by Procedure B. The second reading was made at 21 hours in each instance. The data are shown in Table III. (Four samples were analyzed by a modified procedure and are therefore not included in Table III.)

As a further test of the reliability of this procedure, determinations of the isomer distribution in five 24 hour urine samples were made by one of us (V. H.) and independently by another individual[3] who had been trained in the method. The source of the urine samples and the results of the other analyst were unknown until each had completed the five runs. The results of this experiment are shown in Table IV, from which it is evident that the agreement was reasonably satisfactory.

<div align="center">DISCUSSION</div>

Isomer analysis by Procedure B is regarded as less liable to error because the factors of time and temperature are not so delicately balanced as they are in Procedure A. Using the latter, we have found that consistent results are obtained only when 1½ hours of refrigeration are used, 1 hour or 2 hours being unsatisfactory. With Procedure B, however, much more latitude is allowed both in the period of freezing and in the time thereafter at which the second reading may be made. Data bearing on these points are described in detail in a separate report (6). This report also includes studies of the coprecipitation phenomenon which is the basis of the fluorescence quenching and hence of the method of isomer analysis; additional data relating to the determination of isomers added to various samples of urine and urine extracts are also given in this report.

The following precautions and reservations may be noted, with respect to the methods described above.

The instrument used for fluorescence measurement must be accurate at relatively low levels of fluorescence intensity. Thus, if the initial reading is 200, a large variation of the final reading at certain levels, *i.e.* from 60 to 45, would give a difference of only 6 per cent (200/60 = 3.33 = 55 per cent copro III; 200/45 = 4.44 = 49 per cent copro III). On the other hand, a small variation from 10 to 5 in the final reading means a difference of 10 per cent (18 *versus* 8 per cent copro III). The instrument should be restandardized as often as necessary to insure accuracy.

The instrument should be so calibrated that the highest reading (*i.e.*

[3] We are indebted to Mr. Donald Sutherland for these determinations.

300 on the Klett apparatus) represents a concentration of about 60 γ per cent. Readings above 300 should be diluted immediately with previously prepared 35 per cent acetone, since at high concentrations coproporphyrin I precipitates rapidly even at room temperature. Crude measurement of the fluorescence in the 3.5 cc. acetone permits one to predict the reading to be expected after dilution with water. If the concentration in 3.5 cc. is too high, more acetone is added, followed by the proper amount of water.

Reagents used for chromatography and isomer analysis should be free of any blue fluorescence in ultraviolet light. We have found that a weekly distillation of reagents is adequate in this regard.

The metal complexes of coproporphyrin may give rise to difficulty. The zinc complex is readily split by hydrochloric acid, and any zinc coproporphyrin present in the native urine or formed prior to esterification because of contact of the urine with soft glass will be included as the free porphyrin in the total which is subjected to isomer analysis. The zinc complex may also be formed if the ester comes in contact with zinc, either due to use of soft glass or contaminated reagents, such as methyl alcohol from metal drums. This complex separates from the free coproporphyrin methyl ester on the $CaCO_3$ column, remaining well above it under the conditions described, and exhibiting a yellow or orange rather than a pink or red fluorescence in ultraviolet light. The copper complex is much more difficult to detect, since it does not exhibit fluorescence in ultraviolet light and is not split by HCl. Fortunately, formation of the copper complex in the native urine or even on long standing in soft glass containers appears to be rare so far as we can determine. The copper complex may be split by solution in concentrated H_2SO_4.

It is essential that the Evelyn tubes be tightly corked to prevent evaporation of acetone. For a time, we used beeswax to seal the tubes, but this is unnecessary if sufficient care is given to fitting the corks carefully.

Since the total porphyrin concentration must lie in the 20 to 50 γ per cent range (2, 6), there is no reason why the method cannot be applied to less than 2 γ of total porphyrin by appropriately reducing the volume and using specially selected small tubes. Since we have not had occasion to do this, we cannot say whether or not Fig. 4 would apply without modification.

This method has been applied to many hundreds of urine samples obtained from individuals having a variety of diseases, notably those of the liver and blood-forming organs. The results of these studies will be made the subject of separate communications.

SUMMARY

1. Procedures not entailing crystallization are described for the quantitative determination of the urinary coproporphyrin isomers I and III.

Partially purified solutions containing 2 to 5 γ of total coproporphyrin are required for the isomer analysis.

2. The procedures depend upon a marked difference in fluorescence stability of the two porphyrin methyl esters in 30 to 35 per cent aqueous acetone in the cold, that of coproporphyrin III being relatively much more stable.

3. The validity of the method has been confirmed by the recovery of added amounts of each of the coproporphyrin isomers with an average error of less than 3 per cent, also by isolation of the crystalline porphyrin esters from large volumes of the same urine samples, with subsequent identification of the predominant isomer type by means of ester melting point determination.

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VARIATIONS OF THE INDIVIDUAL BLOOD PLASMA AMINO ACID NITROGEN LEVEL

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Several papers (1-3) have been published recently on the subject of the amino nitrogen of human blood plasma as determined by Hamilton and Van Slyke's ninhydrin method (1).

The values reported for the blood plasma filtrates of persons in the post-absorptive state vary from 3.35 to 5.00 (1), 2.3 to 7.3 (2), and 3.37 to 4.97 (3) mg. of amino nitrogen per 100 ml. of plasma.

It has also been reported (3, 4) that significant differences of the amino nitrogen level are found when samples are taken from the same person on different days.

The above reports do not show whether each individual develops his own average amino nitrogen level or passes through the full range of observed values. Only a large number of analyses carried out on blood samples from a large number of persons can absolutely answer this question. However, the results of a limited number of analyses can be dealt with by means of statistical techniques.

We have, therefore, endeavored to follow the fluctuations of the amino nitrogen contents of human blood plasma by Van Slyke's ninhydrin technique for periods of more than 3 months.

The blood samples were taken during the summer and autumn months of 1944 from two distinctly different groups of people: (1) presumably healthy individuals (two men and three women) of ages between 21 and 38 years; (2) a group of twelve males, mental patients, physically well, of ages between 29 and 77 years, who were under strict dietary control for well over 1 year as participants in a nutritional project being conducted at Elgin. Differences due to environment or working conditions can, therefore, be minimized as factors affecting the data reported below.

Method

Samples were taken before breakfast and the oxalated plasma treated according to Hamilton and Van Slyke's ninhydrin method (1). No correction was made for plasma urea concentration, since no urea concentration of more than 18 mg. of urea nitrogen per 100 ml. of plasma had been found.

The average difference between duplicate determinations of plasma was 1.5 per cent or 0.064 mg. per cent of amino nitrogen. The analytical results were checked daily by including a sample of tyrosine or tyrosine and glutamic acid with the plasma filtrates. Because recoveries of amino nitro-
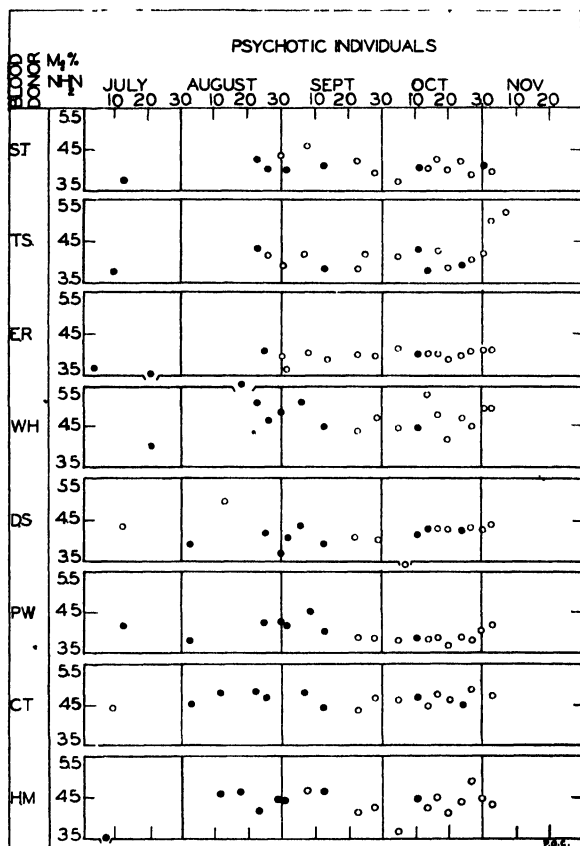
FIG. 1, A

FIG. 1. Plasma amino nitrogen levels obtained from the plasma of individuals. ● represents the average of determinations on at least two separate plasma filtrates; ○ the average of determinations on a single blood filtrate; * represents female subjects.

gen from these amino acids were consistently accurate to better than 1 per cent, two of the more experienced operators of the Van Slyke manometer apparatus did single determinations on many of the later samples (see Fig. 1).

The data reported in Fig. 1 were first used to determine the average of each individual (Table I, Column b). Then the deviations from this aver-

age and the optimum standard deviation estimate, s, were computed, $s = \sqrt{\Sigma (X-\bar{X})^2/(n-1)}$, where X is the analytical result, $\bar{X}$ the arithmetical mean of all the data for one individual, and n the number of determinations. The confidence limits of the individual averages were then determined for the 95 per cent probability point (Table I, Column e)
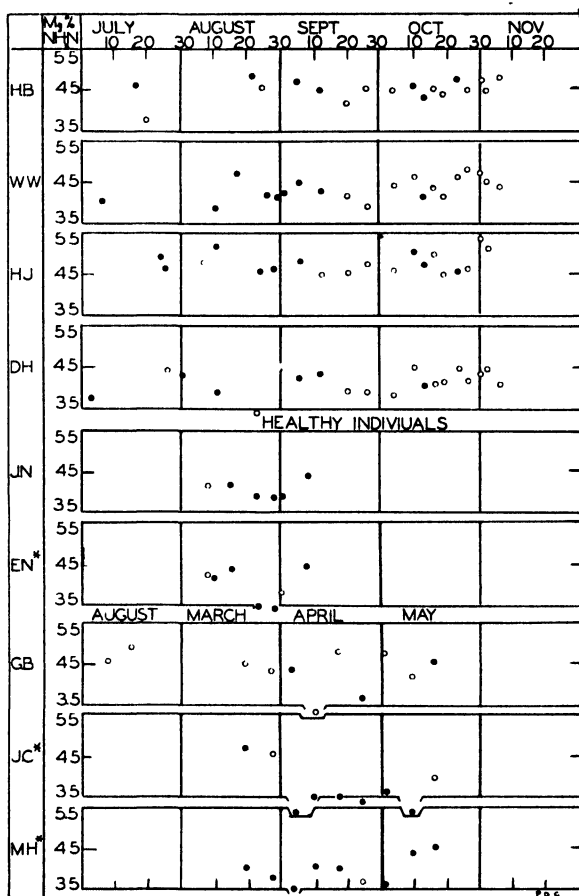
FIG. 1, B

according to the equation $P_{95\%} = \pm\, ts/\sqrt{n}$, where t is the probability factor for the 95 per cent point, $n-1$ the degrees of freedom (6, 5), and s the optimum standard deviation estimate (Table I, Column d). As an example the average of eighteen determinations carried out on subject C. T. is 4.65 mg. per cent with a 95 per cent confidence limit of ±0.07. There is, therefore, only 1 chance in 20 that the "true" average of this person's

amino nitrogen is outside the limits 4.72 and 4.58 mg. per cent.   There is an even chance that any fasting sample taken from this subject will show

TABLE I

Results of Statistical Computations

The results in Columns b, d, and e are expressed in mg. per cent of amino acid nitrogen.

Subject	No. of determinations, n (a)	Average $\bar{X}$ (b)	$\Sigma d^2 = \Sigma(X - \bar{X})^2$ (c)	Optimum S.D. estimate, s (d)	Confidence limits (95 per cent probability) of average (e)
Healthy					
G. B.	11	4.36	2.410	0.490	0.31
J. C.	9	3.71	2.841	0.595	0.41
M. H.	9	3.94	1.327	0.406	0.28
J. N.	6	4.04	0.209	0.203	0.17
E. N.	7	3.98	1.031	0.415	0.33
Psychotic					
S. T.	18	4.08	0.725	0.201	0.09
T. S.	18	4.18	2.378	0.374	0.18
E. R.	18	3.96	0.472	0.166	0.08
W. H.	18	4.75	2.907	0.413	0.20
D. S.	19	4.17	1.830	0.310	0.15
P. W.	18	4.00	0.733	0.208	0.10
C. T.	18	4.65	1.381	0.149	0.07
H. M.	18	4.37	1.806	0.325	0.16
H. B.	18	4.45	1.100	0.254	0.13
W. W.	20	4.28	1.415	0.273	0.14
H. J.	18	4.73	1.111	0.255	0.13
D. H.	19	4.08	1.346	0.273	0.13
17 individuals	.. ..	4.22	1.472	0.297*	0.18†

$$* \text{ Optimum standard deviation estimate} = \sqrt{\frac{\Sigma \text{ (individual average} - 4.22)^2}{17 - 1}} .$$

$$† \text{ 95 per cent probability} = t_{16}s/\sqrt{n} = \frac{2.1 \times 0.297}{\sqrt{17}} ; t_{16} = \text{the probability factor}$$

for 16° of freedom (5).

a deviation from 4.65 mg. per cent by more or less than 0.149 mg. per cent (Table I, Column d).

The average of all the 262 determinations was found to be 4.26 mg. per cent.   If, however, the seventeen individual averages (Table I, Column b) are averaged, the more reliable total average of 4.22 mg. per cent is found.

The optimum standard deviation of the individual average from 4.22 mg. per cent is computed to be 0.297. Any specific sample taken differs, of course, from the donor's average value and this variability has been estimated to be 0.307 mg. per cent from the relation

$$s = \sqrt{\frac{\Sigma(X_1 - x_1)^2 + \cdots \Sigma(X_{17} - x_{17})^2}{(n_1 - 1) + \cdots (n_{17} - 1)}}$$

If there was on the average no difference between donors, the standard deviation of the donor's average from 4.22 mg. per cent would be $0.307/\sqrt{15.3} = 0.078$ mg. per cent, where 15.3 is the average number of determinations per donor. Actually, as shown above, the value is 0.297, indicating that there is more difference between donors than would be expected from chance causes alone.

The question remains whether it is likely that any individual would cover the full range of possible amino nitrogen values, or whether a certain smaller range of values is preferred. In other words, statistically expressed, are all the observed values taken from the same source or from different sources?

Bartlett's χ^2 test (7) has been used here.

$$\text{Crude } \chi^2 = 2\,303[(\log V_o) - (\Sigma df)(\log V)]$$

where $V = s^2$, $V_o = (\Sigma (df)(V))/\Sigma df$, $df = (n - 1)$. .

Both the crude and the corrected value of χ^2 proved that the samples were not taken from the same source. A similar result was obtained through the analysis of the variance of the individual data and variance of the means.

It is physiologically significant that certain individuals have smaller variations from their mean values than other individuals who show wider variations. The causes of these individual differences in stability of plasma amino acid concentration remain to be investigated, but they may be related to the subject's emotional stability (4), since it has been shown that pharmacological stimulation of the sympathetic nervous system causes a marked drop in plasma amino nitrogen.

SUMMARY

1. Covering a period of about 3 months, an average of 15.3 samples of fasting blood has been taken from each of seventeen persons (three women and fourteen men) and the plasma amino nitrogen has been determined by the ninhydrin manometric method. Fig. 1 shows these data plotted against time.

2. The average amino nitrogen value of all the above 262 determinations was 4.26 mg. per cent.

3. Bartlett's χ^2 test applied to the above figures and analysis of the vari-

ances showed that it is very improbable that the above data were all drawn from the same source. It is, therefore, concluded that each individual develops his personal average amino nitrogen level and any given sample fluctuates around this individual average.

4. The true average amino nitrogen value is, therefore, more correctly determined by averaging the individual averages. This gave the average value of 4.22 mg. per cent amino nitrogen. In computing the confidence limits of this value, it was determined that there are 19 chances in 20 that the true average would lie within the limits of 4.07 and 4.37 mg. per cent.

5. The basal plasma amino nitrogen values of an individual do not seem to fluctuate greatly from day to day, but to develop upward or downward trends, which last for a limited number of days.

6. The range of amino nitrogen in the plasma is an individual function that remains fairly constant from month to month.

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THE ESTIMATION OF PARATHYROID HORMONE ACTIVITY BY ITS EFFECT ON SERUM INORGANIC PHOSPHORUS IN THE RAT*

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The activity of parathyroid hormone preparations is commonly estimated by the method of Collip and Clark (1, 2), which has been adopted as a standard procedure in the United States Pharmacopoeia. The U. S. P. unit is defined by this method as 0.01 of the amount of parathyroid hormone required to raise the serum calcium of a 10 to 12 kilo dog 1 mg. per cent within 16 hours after subcutaneous injection of the material under test. The estimate is based on the specified response of the animal, without reference to a standard, on the assumption that the response is directly proportional to the dose. Miller (3) and Bliss and Rose (4) have shown that the method of estimating the serum calcium in the dog has serious limitations, but that by employing suitable experimental designs (involving fairly large numbers of dogs and much time) satisfactory assays of parathyroid hormone preparations may be made in terms of a standard preparation employed in parallel with the unknown.

In either form, the dog serum calcium method is unsuitable for rapid and reasonably accurate estimations of the potency of the numerous fractions obtained during work on the isolation and purification of the parathyroid hormone. From the standpoint of uniformity of material, cost, ease of maintenance, and the requirements of number for a single estimation, the rat offers every advantage over the dog. The object of this paper is to show that male white rats weighing from 150 to 250 gm. exhibit a measurable fall in serum inorganic phosphorus 3 hours after the subcutaneous injection of parathyroid hormone, that the relation between the fall in serum inorganic phosphorus and the logarithm of the dose of hormone is substantially linear over the range 12.5 to 100 U. S. P. units, and that this

relationship may be used to estimate the activity of parathyroid hormone preparations in terms of a previously determined standard dose-response curve, or in terms of a standard preparation tested in parallel, with an accuracy at least as great as that of the dog serum calcium method and with far greater convenience.

<div align="center">EXPERIMENTAL</div>

Materials and General Methods—Male albino rats of the Sprague-Dawley strain were used. They were fed a standard ration of Purina dog chow and were allowed to become accustomed to the laboratory and the diet for at least a fortnight before use. Except when otherwise indicated, they were not fasted before use, but during the experiments they were allowed only water. Since the animals were used in the late morning or early afternoon hours, they were considered to be in the postabsorptive state. Blood samples were taken without anesthesia, the animal being temporarily restrained in a small rat holder. The blood (0.6 ml.) was "milked" from the cut end of the tail into 1 ml. centrifuge tubes. The tubes were centrifuged for 10 minutes, and 0.2 ml. samples of the serum were pipetted into 6 ml. of 10 per cent trichloroacetic acid in 15 ml. centrifuge tubes. A 5 ml. aliquot of the protein-free supernatant fluid was taken for the determination of inorganic phosphorus by the method of Fiske and Subbarow (5), modified for the Evelyn photoelectric colorimeter.

Parathyroid extract (Lilly) was used as a standard preparation throughout the experiments. In constructing a dose-response curve the same lot of material was used for all of the observations. For doses of less than 100 units (1 ml.), the extract was diluted with 0.9 per cent sodium chloride solution so that in every instance the volume of the test injection was 1 ml. Except when otherwise indicated, the test solutions were injected subcutaneously in 0.5 ml. portions on each side in the lumbar region. The injections were made immediately after the initial blood sample was taken, and the animals were then placed in individual cages where they remained quietly until the time for the final blood sample. In the course of the work no animal was used more often than every 2nd and, in most instances, every 3rd day.

The animals first used to determine the response to the hormone and to establish the relation between the response and the amount of hormone injected are designated in Tables I to VII and in Fig. 1 as Group I, and those used later to check the initial observations and to develop the final form of the method of estimation are designated as Group II. Each group behaved consistently, but the animals of Group I were less sensitive, especially to low doses of the hormone, and tended to exhibit a marked rise in serum inorganic phosphorus in control experiments in which no hormone

was given. The reason for these differences is not clear, but on the basis of changes in the behavior of rats of the same strain in other experimental work being carried on in the laboratory at the time it is suspected that alterations of the ration due to wartime conditions may have been responsible.

Effects of Parathyroid Hormone on Serum Inorganic Phosphorus in the Rat—Table I summarizes observations made upon six series of fed rats: three sets of control animals given injections of 1 ml. of 0.9 per cent sodium chloride solution, and three others given 50 units of parathyroid hormone

<div align="center">TABLE I</div>

Effect of Parathyroid Hormone on Serum Inorganic Phosphorus of Fed and Fasted Rats

No. of animals	State	Dose	Route of injection	Serum inorganic phosphorus, mg per cent					
				Initial	Difference from initial				
					1 hr	2 hrs	3 hrs	4 hrs.	5 hrs
				Group I					
		units							
4	Fed	0	Subcutaneous	8 40	+0 65	+1 32	+1 47		
8	"	50	"	9 23	−1.18	−1 45	−1 72		
				Group II					
3	Fed	0	Subcutaneous	9.72	−0 67	−0 45	−0 79		
18	"	0	"	9 28			−0.49 ±0.14		
16	"	50	"	9 15			−1.73 ±0 11		
2	Fasted	0	"	8.17	+0 60	+0 08	+0 17		
9	"	50	"	8.12	−0 46	−0 06	−0.01		
22	"	50	"	8.30			−0 44 ±0 19		
4	"	50	Intraperitoneal	8 07	−0 25	+0 01	+0 42	+0 82	+1.11

subcutaneously. Serum inorganic phosphorus was determined initially, and either hourly for 3 hours after the injection or only at the 3rd hour. Similar observations were made upon three series of rats fasted for 24 hours, and the effects of intraperitoneal injection of 50 units of the hormone were studied in a small group of four rats.

The expectation that a 24 hour fast might lead to more consistent responses to the parathyroid hormone was not realized. The responses were irregular, small, and transitory. Intraperitoneal injection of the hormone into fasted rats produced a modest fall followed by a sustained rise in serum inorganic phosphorus during the next 4 hours. Fasted rats seemed, from

these observations, to be less suitable for estimating parathyroid activity than fed rats. The differences in the responses are interesting and suggestive, but their detailed study was not relevant to this work, and it was therefore deferred.

In the fed control animals the serum inorganic phosphorus rose (Group I)

TABLE II

Effect of Graded Doses of Parathyroid Hormone, 3 Hours after Subcutaneous Injection, on Serum Inorganic Phosphorus of Fed Rats

Dose		No. of observations	Serum inorganic phosphorus, mg. per cent	
			Mean initial level	Mean fall
Group I				
units	*log units*			
12.5	1.0969	5	8.52	−0.51	−0.20*
25	1.3979	21	8.98	0.94	1.02
50	1.6990	129	9.26	1.18	1.12
100	2.0000	8	9.18	1.74	1.72
Group II				
12.5	1.0969	24	9.35	0.70	0.60
25	1.3979	36	9.06	1.04	1.09
50	1.6990	121	9.16	1.65	1.64
100	2.0000	22	9.00	1.84	1.91

Relevant statistics for relation between the adjusted fall in serum inorganic phosphorus (y) and the log dose of parathyroid hormone (x):

$$\bar{x}_a = 1.6070 \qquad \text{Reduced } [y^2] = 71.1120$$
$$\bar{y}_a = 1.4478 \qquad s^2 = 0.3592, \ n = 198$$
$$[x^2] = 12.2394 \qquad V(a_a) = 0.001769$$
$$[xy] = 19.2574 \qquad V(b) = 0.02934$$
$$[y^2] = 102.3323 \qquad V(b)/b^2 = 0.01185$$
$$b_a = 1.5734 \quad \text{Curvature (mean square) } 0.4604, \ n = 2, \ F \ 1.282$$

Standard curve (adjusted for $w = 9.15$: $Y = 1.4478 + 1.5734 \ (x - 1.6070)$.

* The values in bold-faced type were obtained by adjusting all responses to the same initial level, 9.15 mg. per cent.

or fell slightly (Group II) during the 3 hours after injection. In the fed animals given 50 units of parathyroid hormone there was a large and fairly consistent fall in serum inorganic phosphorus 3 hours after the injection in both groups. A few observations not recorded in Table I showed that the effect had reached its maximum by the 3rd hour.

The relation between the amount of parathyroid hormone injected and the change in serum inorganic phosphorus occurring 3 hours after the

injection was determined in two groups of fed rats.   The data are summarized in Table II and Fig. 1.   In Group I a satisfactory relationship between the log dose of hormone (in units) and fall in serum inorganic phosphorus (in mg. per cent) was not obtained.   When the observations were repeated 2 years later with adequate numbers of animals, a good linear relationship was obtained between the log dose of hormone and fall in serum inorganic phosphorus over the whole range between 12.5 and 100 units.   Table II gives the data for the initial observations (Group II) which were used for
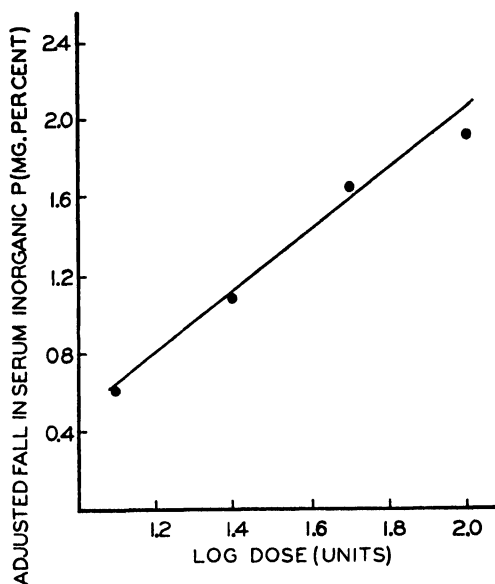
FIG. 1. Dose-response curve for parathyroid extract in terms of fall in serum inorganic phosphorus (adjusted to constant initial level), in mg. per cent (y) against the logarithm of the dose, in units (x).   Formula of regression line, $y = 1.4478 + 1.5734 (x - 1.6070)$.

subsequent estimations of the activity of preparations of the hormone (6) plus the extended observations made in the course of additional checks on the responses of the animals.   No significant differences (such as those observed between Groups I and II) appeared between these two portions.

The preponderance of observations on the response to 50 units of the hormone is the result of a routine practice of testing all new rats before they were used in the estimation of unknown parathyroid hormone preparations, since in the preliminary work it was found that some rats fail to respond to the hormone.   No animal was used if its serum inorganic phosphorus fell by less than 0.5 mg. per cent in response to 50 units of the hormone.   These

data also provided an opportunity for examining the relation between the initial level of serum inorganic phosphorus (which tended to be variable) and the fall in response to 50 units of the hormone.   Since there seemed to

TABLE III

Regressions within Each Dose of Initial Level of Serum Inorganic Phosphorus on Fall in Serum Inorganic Phosphorus in Response to Parathyroid Hormone

The initial level (w), in mg per cent, is coded, 7 having been subtracted from each observed value; fall (y), in mg. per cent.

	Doses of parathyroid hormone				
	12 5 units	25 units	50 units	100 units	Totals
$S(w)$	56 40	74.05	261.25	43.99	435.69
$S(y)$	16 84	37.39	199 30	40.38	293 91
$S(w^2)$	141 1638	170 0363	643 5147	95 4277	1050 1425
$S(y^2)$	19 1334	57.0267	387 9908	87.7778	551 9287
$S(wy)$	43 2001	87 7392	465 5617	87 1292	683 6302
$[w^2]$	8 6238	17 7196	79 4522	7 4677	113 2633
f	24	36	121	22	203
$[wy]$	3 6261	10 8300	35 2549	6 3876	56 0986
$[y^2]$	7 3173	18 1930	59 7223	13 6622	98 8948
b	0.4205	0 6112	0 4437	0 8554	
Reduced $[y^2]$	5 7925	11 5737	44 0797	8 1982	69 6441
s^2	0 2633	0 3404	0.3704	0 4099	
n	22	34	119	20	
Adjusted $[y^2]$ (for $b = 0$ 5)	5 8472	11.7929	44 3304	9 1415	71 1120

Combined values $[w^2]_c = 113.2633; [wy]_c = 56\ 0986; b_c = 0\ 4953; V(b_c) = 0\ 003153,$ $\bar{w}_c = 2.15 \pm 0\ 05$

Observed values of y may be adjusted to the same initial level by applying the equation, $y_a = y_o - 0\ 5\ (w_o - 9\ 15)$.

TABLE IV

Analysis of Variance of Data of Table III to Determine Significance of Differences between Slopes at Each Dose Level

Source of variation	Degrees of freedom	Mean square	F
Effect of combined slope .	1	27 7853	
Variation in slope	3	0 4884	1 37
Deviations from individual slopes..	195	0 3571	

$F = 0.4884/0\ 3571 = 1.37$ (F for 5 per cent level of significance $= 2.65$).

be a significant relation between these quantities at 50 units, the data of Group II were examined in detail for this effect at each dose level.

Table III presents the data from the 203 observations of Group II for the regressions within each dose of fall in serum inorganic phosphorus $(y,$

in mg. per cent) on initial level (w, in mg. per cent), and in Table IV an analysis of variance for estimating the significance of the differences between slopes is presented.   An effect of initial level upon fall was seen at each dose level, and the analysis presented in Table IV showed that the differences in slopes (b) for the different doses were not significant, so that the effect was substantially the same at each dose level.   It was therefore possible to calculate a combined slope (b_c) which could be used to adjust the observed values of the fall in serum inorganic phosphorus to the values they would have if the initial level were the same in each instance.   This was done by means of the equation, $y_a = y_o - b_c(w - \bar{w})$, where y_o is the observed, and y_a is the adjusted fall in serum inorganic phosphorus (in mg. per cent), b_c is the combined slope of the regression of initial level on fall, w is the observed initial level of serum inorganic phosphorus (in mg. per cent), and $\bar{w}$ is the mean initial level observed for the 203 observations of Group II. The combined slope, b_c, is 0.4953 ± 0.0561.   No serious error is introduced if the value 0.5 is taken for convenience.   The value of $\bar{w}$, from Table. III, is 9.15 mg. per cent.

Before the regression of fall in serum inorganic phosphorus on the log dose of hormone was calculated, all of the values were adjusted to the same initial level of serum inorganic phosphorus by means of the relation set forth in the foregoing paragraph.   This has the effect of eliminating errors due to variation in the initial levels of serum inorganic phosphorus, and brings about a corresponding reduction in the errors of the estimates of relative potency of unknown parathyroid preparations.

Estimation of Relative Potency of Parathyroid Hormone Preparations. Short Procedure with Predetermined Standard Dose-Response Curve—The preparation to be tested is given to six animals at two dose levels selected so that as nearly as possible the responses will be restricted to the central portion of the standard curve.   The doses of the unknown (measured in mg. of nitrogen) are put into the same terms as the standard by converting them to their logarithmic equivalents in U. S. P. units on the basis of 100 units per mg. of nitrogen (which is the average potency of the commercial preparation used as a standard).   The fall in serum inorganic phosphorus 3 hours after the injection is determined in each of the six rats and is corrected to the initial preinjection level of 9.15 mg. per cent, as described in the foregoing section.   The log ratio of the potencies of the unknown and the standard is then calculated by the standard procedure modified for the use of a predetermined standard dose-response curve.[1]   These operations are illustrated in detail in a representative estimation made upon a preparation obtained in the course of work on the isolation of the parathyroid

[1] Bliss, C. I., personal communication.

hormone ((6) Table I, B acid, acetone Fraction D1). The experimental data and the derived statistics are presented in Table V.

If the unknown sample produces the same biological response as the standard, a plot of the responses to the unknown should yield a line substantially parallel to the standard line, so that a combined slope will fit both sets of observations. On the log dose scale the horizontal distance between the two parallel lines is the log ratio of the potencies, M, which may be calculated from the equation

$$M = \bar{x}_s - \bar{x}_u + \frac{\bar{y}_u - \bar{y}_s}{b_s} \tag{1}$$

TABLE V

Data for Estimation of Potency of Unknown Parathyroid Hormone Preparation by Method of Comparison with Predetermined Dose-Response Curve

Rat No.	Dose		Serum inorganic phosphorus, mg. per cent		
			Initial	Fall	Adjusted fall, y
	mg. of N	*log units, x*			
97	0.2	1.3010	8.86	0.86	0.915
98	0.2	1.3010	9.66	1.66	1.405
99	0.2	1.3010	9.37	1.37	1 260
100	0.5	1.6990	10.51	3.15	2.470
101	0.5	1.6990	9.95	2.31	1.910
102	0.5	1.6990	8.95	1.87	1.970

Derived statistics, number of observations (N) = 6.

$S(x)$, log dose (units) = 9.0000 $[xy] = S(xy) - \bar{x}_u S(y) = 0.55123$
$S(x^2)$ = 13.7376 $b = [xy]/[x^2] = 2.320$
$S(y)$, fall (adjusted) = 9.930   Reduced $[y^2] = [y^2] - b[xy] = 0.31575$
$S(y^2)$ = 18.02875   Adjusted $[y^2] = b_s^2[x^2] - 2b_s[xy] + [y^2]$,
$S(xy)$ = 15.44623   = 0.4483
$\bar{x}_u$, mean log dose = 1.5000 $s^2 = $ adjusted $[y^2]/(N - 1) = 0.08966$
$\bar{y}_u$, mean adjusted fall = 1.6550 $V(a_u) = s^2/N = 0.01494$
$[x^2] = S(x^2) - \bar{x}_u S(x) = 0.2376$ $b_s = $ slope of standard dose-response
$[y^2] = S(y^2) - \bar{y}_u S(y) = 1.59460$ curve = 1.57

Expected $Y = 1.2794$ at $X = 1.500$, $M = 0.2387 \pm 0.0834$.

in which $\bar{x}_s$ is the mean log dose for the standard, $\bar{x}_u$ that for the unknown, $\bar{y}_s$ is the mean response to the standard, $\bar{y}_u$ that to the unknown, and b_s is the slope of the standard dose-response curve. The standard error of M, s_M, is estimated by the equation

$$s_M = \frac{1}{b_s} \sqrt{V(a_u) + V(a_s) + \frac{(\bar{y}_u - \bar{y}_s)^2 V(b_s)}{b_s^2}} \tag{2}$$

in which $V(a_u)$ and $V(a_s)$ are the variances of the mean response of unknown and standard respectively about the parallel dose-response curves, $V(b_s)$ is the variance of the slope of the standard dose-response curve, and the other terms have the same significance as in equation (1). The standard error of the relative potency may be computed directly, when s_M is small, by

$$\text{s.e of relative potency} = 2\,303\,s_M\,(100\text{ antilog }M) \qquad (3)$$

The values for $\bar{x}_s$, $\bar{y}_s$, b_s, $V(a_s)$, and $V(b_s)/b_s^2$, computed from the data for the standard curve, are presented in Table II. The correction for the use of the predetermined dose-response curve involves, first, testing to see whether the slope of the curve of the responses for the unknowns is not significantly different from the slope of the standard dose-response curve. In the example cited, the slope for the unknowns is 2.32, and its standard error is ±0.58; it is clearly not significantly different from the predetermined slope, 1.57. The correction may therefore be applied by making an estimate of $[y^2]$ not as usual in terms of the sum of the squares of the distances of the observed y from the regression line for the experimental points, but in terms of the sum of the squares of the distances of the observed y from that line rotated around its mid-point to make its slope the same as that of the standard curve   This is done by means of the equation

$$\text{Adjusted }[y^2] = b_s^2[x^2] - 2b_s[xy] + [y^2]$$

where b_s is the slope of the standard curve. The adjusted $[y^2]$ is then used to calculate s_u^2 and to estimate $V(a_u)$.

Substitution of the relevant figures from Tables II and V in equation (1) yields

$$M = 1\,607 - 1\,500 + \frac{1.6550 - 1\,4497}{1.57} = 0\,2378$$

and, from equation (2),

$$s_M = \frac{1}{1\,57}\,\sqrt{0\,01507 + 0\,0017 + (1\,655 - 1\,4497)^2(0.0118)} = 0\,0836$$

The estimated value of $M \pm s_M$ is therefore 0.2378 ± 0.0836. The potency of the unknown, in per cent of the standard, is found by multiplying antilog M by 100: 1.73 × 100 = 173 per cent. From equation (3) the error of the estimate is (2.303) (0.0836) (173) = ±33 per cent. On the basis of the standard preparation assaying 100 U. S. P. units per mg. of nitrogen, the estimated potency of the unknown is therefore 173 ± 33 units per mg. of nitrogen.

This is fairly representative of the results obtainable in the routine use of

the method for the estimation of parathyroid activity in numerous fractions obtained during the isolation and partial purification of the parathyroid hormone (6). In the example cited, the slope of the regression line of the observed points deviated rather more than usual from the slope of the standard curve, and the error of the estimate is correspondingly large. It is realized that the errors of the method are appreciable, and that the method cannot be relied upon to detect small differences in relative potency. But since even the best of contemporary parathyroid hormone preparations are crude, large increases in potency may still be anticipated in the course of purification, and the method can be relied upon to detect such increases.

Estimation of Parathyroid Activity with Standard and Unknown Preparations Employed in Parallel—Bliss and Rose (4) have shown that by the application of proper experimental designs, the "Latin square" arrangement or the simpler "symmetrical pairs" method of Yates, the assay of parathyroid hormone by the dog serum calcium method can be improved. Such designs, correctly applied, increase the precision of the estimates by segregating differences due to animals, days, etc., and may yield information leading to improvement in the assay itself. Such an instance is the discovery by Bliss and Rose that the final level of serum calcium in the dog provides as good a measure of the effect of parathyroid hormone as the increase in serum calcium. Alternatively, these designs may provide greater insight into the mechanism of the biological activity that is being studied.

In order to ·see how well the rat serum inorganic phosphorus method compares with the dog serum calcium method in a more rigorous experimental design, a single trial assay by the symmetrical pairs method of Yates was undertaken. As the design was applied, each rat was injected at an interval of 5 days, once with one treatment and once with another. Four treatments were used, U_1, U_2, S_1, S_2, the letters U and S referring to unknown and standard, and the subscripts 1 and 2 to the different doses. In this instance, the unknown was the standard itself in the same dilution. Each treatment was paired equally often with every other, sò that six rats were required to cover all possible combinations. Because of the interval between successive treatments, each pair of treatments was applied at the same time in reverse order to six additional rats.

The data for the complete set of twelve rats, tested as described, are presented in Table VI. The statistical calculations were carried out so as to recover interpair information, as described by Bliss (7), and will not be presented in detail. The calculations were made in terms of the fall from the initial level of serum inorganic phosphorus, and the adjustment for differences in the initial level was determined by covariance. Differences in body weight were not adjusted.

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TABLE VI

Data on Responses of One Set of Twelve Rats to Parathyroid Hormone in Yates Symmetrical Pairs Assay

</div>

Doses, 25 U S. P units (U_1 and S_1) and 80 U S P units (U_2 and S_2); volume of injection in each instance, 1 ml.

Pair No.	Treatment		Body weight		Serum inorganic phosphorus, mg per cent			
	1st period, June 25	2nd period, June 30			Initial		Fall from initial	
			June 25	June 30	June 25	June 30	June 25	June 30
			gm.	*gm.*				
1	U_1	U_2	198	212	8 06	8.32	0 35	1.79
2	"	S_1	216	228	9.37	7.82	1 25	0.29
3	"	S_2	200	204	8.60	8.18	0.66	1.75
4	U_2	S_1	212	220	9 06	7.19	1.87	0 71
5	"	S_2	196	208	9.37	8 80	2 01	2.11
6	S_1	"	182	196	9.06	9 72	1 70	1.72
7	U_2	U_1	193	216	10.38	9.72	2 06	0 98
8	S_1	"	196	205	10.10	9 01	1.24	0.58
9	S_2	"	188	200	9.95	9 32	2 19	1.44
10	S_1	U_2	193	212	9.37	10 23	1 31	1.86
11	S_2	"	191	208	10.01	9 52	1 95	2.16
12	"	S_1	200	220	9.65	9 52	2 01	0.78

<div align="center">

TABLE VII

Analysis of Variance in Parathyroid Assay Based on Fall in Serum Inorganic Phosphorus in Rat (Yates) (Symmetrical Pairs, Analyzed to Segregate Interrat Error)

</div>

Variation due to	Degrees of freedom	Sum of squares	Mean square
Days... .	1	0.2460	
Rats, from replicated rows .	6	0.7805	0 1301
Doses, ignoring groups	3	6 2409	
Grouping, eliminating dose	5	0 3035	0.0607
Intragroup error ..	8	1.1090	$0.1386 = s_1^2$
Total . .	23	8 6799	

Treatment effects:

(1) Differences between S and U = T_a = -0.75

(2)   "   due to doses (slope) = T_b = 12 19

(3)   "   "   " parallels = T_c = 0.79

"Just significant difference" = c = (approximately) 4.206 ($P = 0.05$, $t = 2.306$)

Log interval between doses = z = 0.5052

" ratio of potencies = $i\, T_a/T_b$ = $\bar{1}.9689$

Confidence limits ($P = 0\ 05$),

$$X_L = \frac{i(T_a\, T_b \pm c\sqrt{T_a^2 + T_b^2 - c^2})}{(T_b^2 - c^2)} = \frac{+0.1509}{\bar{1}.7786}$$

A summary of the results of the analysis is presented in Table VII. It is seen that in this experiment the error between rats (grouping, eliminating dose, 0.0607) is smaller than the error within rats (intragroup error, 0.1386). Thus no adjustment of the treatment effects for interrat error is necessary, and the error variance for intrarat error, $s_1^2 = 0.1386$, is used directly in the subsequent calculations. This result is unexpected, and, since it might not occur in additional trials, it is probably advisable to continue segregating the interrat error as a safeguard in future experiments. In the analysis of covariance for the effect of differences in initial level of serum inorganic phosphorus it was found that adjustment for differences' in initial level had little effect on the intrarat error but diminished the interrat error significantly. If this proved to be a consistent effect in additional experiments, an adjustment for interrat error might be made unnecessary by calculating the results in terms of the responses adjusted to the same initial level of serum inorganic phosphorus, just as it was done for the standard dose-response curve described in the foregoing section   In this experiment, however, the slope of the regression of initial level on fall was 0.2 rather than 0.5, the value found for the observations on the standard dose-response curve. If an adjustment for variations in initial level is to be made in experiments in this design, it may be necessary to determine the effects of the procedure on the relation between initial level and fall.

The significance of the treatment effects may be assessed by comparing them with the "just significant difference," c, calculated from the relation, $c^2 = 24 s_1^2 t^2$, in which t is the value, derived from Fisher's (8) table of t, for $P = 0.05$ and for the number of degrees of freedom for s_1^2. (This value of c may be approximate rather than exact.) The treatment effects for differences in potency between the standard and the unknown (T_a), for parallelism of the standard and unknown dose-response curves (T_c), and for the slopes of the dose-response curves, i.e. for the effect of the difference in dose levels (T_b), are calculated by a factorial analysis of the data, as described by Bliss (7). Since there was no difference between the standard and the unknown, T_a should be very much smaller than c. If the regression lines relating dose and response are substantially parallel, T_c should be smaller than c. If the assay has properly discriminated differences in dose levels, T_b should be very much larger than c. Table VII shows that these conditions are fulfilled, and the assay is therefore valid.

The estimate of the log ratio of the potencies, $\bar{1}.9689$, indicates that the potency of the unknown is 93.1 per cent of that of the standard. It is in satisfactory agreement with the known identity of the unknown and the standard. The confidence limits (9), X_L, of 0.1509 and $\bar{1}.7786$ indicate that, in a series of repetitions of the assay, only 1 in 20 would yield an estimate of relative potency lying outside the limits of 142 and 60 per cent.

These limits may, of course, be narrowed by replication of the experiment. In a similar experiment with the dog serum calcium method, with three sets of twelve dogs (4, 8), the estimate of relative potency for an unknown identical with the standard was 120.4 per cent, with confidence limits at $P = 0.05$ of 95 and 160 per cent. The data of the rat experiment suggest that with three sets of twelve rats as good or better estimates might be achieved.

<div align="center">DISCUSSION</div>

The work described in this paper was undertaken in order to devise a suitable method for use in work on the isolation and purification of the parathyroid hormone. Although the serum inorganic phosphorus is a variable easily susceptible to a number of influences, the evidence presented above indicates that in the fed male white rat changes in the level of serum inorganic phosphorus occurring in a short time after the subcutaneous injection of parathyroid hormone are sufficiently consistent to provide reasonably accurate estimates of the amount of hormone injected. The method with six rats at two dose levels against a predetermined standard dose-response curve was at first employed in a form relating percentage fall in serum inorganic phosphorus to log dose of hormone. This procedure was abandoned as unsuitable after the analysis of the relation between initial level and fall in serum inorganic phosphorus showed that the effect of variations in initial level could be eliminated by adjusting the observed falls to a constant initial level, and the absolute fall (adjusted) was therefore used as the measure of the response. The estimate of relative potency of the preparation described in Table V, based upon the percentage fall in serum inorganic phosphorus, was $M = 0.3120 \pm 0.1236$ (205 $\pm$ 58 per cent), as compared with the estimate, based on adjusted fall, of 0.2378 $\pm$ 0.0836 (173 $\pm$ 33 per cent). There is no great difference in the value for relative potency, but in the second instance the error of the estimate is considerably reduced.

The variability of the responses are such that an estimation with a small number of animals and a predetermined dose-response curve can be relied upon only to distinguish fairly large differences in relative potency between different parathyroid hormone preparations. Such a method, however, is immensely useful in following the major steps of purification in the preparation of the hormone. It offers all the advantages over the dog serum calcium method which were cited in the introduction to this paper. In addition, it may offer more reliable estimates of relative potency, since the dog serum calcium method, as it has been employed by most investigators, is based on the assumption that the response is directly proportional to the dose rather than to the logarithm of the dose (3), and this is likely to increase the uncertainty of the estimates of parathyroid activity.

The trial of the Yates symmetrical pairs design shows that the rat serum inorganic phosphorus method is suitable for the proper assay of the parathyroid hormone and is comparable in performance with the dog serum calcium method, as used by Bliss and Rose (4) and by Bliss (7). The data of this experiment were at first analyzed by the original procedure of Yates, as described by Bliss and Rose (4), with results that compared well with theirs. The recent development of a method for segregating interpair information (7) made it seem desirable to apply this method to these data. The information gained on the relation between intrapair and interpair error indicates that, if further trial of the design shows this relation to be consistent, a procedure in a simpler design may be adopted which, because of its ease of replication, may lead to a considerable reduction in the errors of the estimates of relative potency. Even in its present form, however, the assay, which can easily be carried out by a single experienced person, may be most useful in the final critical stages of purification as well as in the routine standardization of the parathyroid hormone.

It is realized that the method in its present form is as yet imperfect. If, as is suspected, the differences in behavior of the rats in Groups I and II are due to differences in the ration, it is possible that a careful study of the effects of the composition of the diet on the response to the hormone may lead to the discovery of conditions in which the sensitivity of the animals and the uniformity of their responses to the hormone can be greatly increased. Such a study may also throw light upon another characteristic of the rats used in this investigation, the high levels of serum inorganic phosphorus both in the fed and in the fasted state. These values were at first regarded with suspicion, and the method was carefully checked and rechecked several times in the course of the investigation. No technical errors were discovered, and the observed values were consistently obtained in the more than 350 rats used in this study. This may be a property of the strain of rats used. If so, it is possible that this "hypoparathyroid" characteristic of the rat is essential to the success of the method.

SUMMARY

Evidence is presented to show that the subcutaneous injection of parathyroid hormone into male albino rats in the fed (postabsorptive) state produces in 3 hours a fall in serum inorganic phosphorus which is directly proportional to the logarithm of the dose of hormone administered (in U. S. P. units). This relation can be used to establish a standard dose-

response curve with which it is possible to carry out simple and rapid estimations of parathyroid activity in preparations derived in the course of chemical work on the isolation and purification of the hormone.

An example is given of an assay employing twelve rats in a more rigorous experimental design, the symmetrical pairs design of Yates, which demonstrates that the rat serum inorganic phosphorus method is comparable in reliability and accuracy with the dog serum calcium method, employed in the same design, for the estimation of parathyroid hormone activity.

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A NEW PREPARATION OF THE PARATHYROID HORMONE*

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The first successful attempt to obtain more active preparations of the parathyroid hormone than those described by Collip and Clark (1) was reported by Ross and Wood (2) in 1942. Their method was based upon an extraction of beef parathyroid glands with hot dilute acid, followed by fractionation of the extract with alcohol and then with ammonium sulfate, and adsorption of the active principle onto benzoic acid. They obtained a series of preparations with a "nitrogen potency," activity in U. S. P. units per mg. of nitrogen, of 200 to 300, 2 to 3 times as active as the best preparations of Collip and Clark. This paper is a report upon another method of securing parathyroid hormone preparations of nitrogen potency 200 to 300 in decidedly greater yields than those achieved by Ross and Wood.

The new method has three principal steps: (1) preparation of a dry, relatively fat-free gland powder; (2) extraction of the gland powder with warm dilute hydrochloric acid; (3) fractionation of the cleared extract with acetone. The procedure yields about 10,000 units per 100 gm. of fresh glands, several times the quantities obtained by previous methods. The activity of the preparations was in each instance estimated by their effect on the serum phosphorus of the rat, as described in the foregoing paper (3).

EXPERIMENTAL

Materials and General Methods—Fresh frozen beef parathyroid glands were stored in a freezer and worked up in small lots as required. U. S. P. acetone and chloroform were used in the defatting of the glands. The acetone used in the fractionations was carefully redistilled. Nitrogen was determined by the micro-Kjeldahl method. The potency of most of the fractions was tested on groups of six rats at two different dose levels, as

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described for the short method of estimation of parathyroid activity in the foregoing paper (3).

Isolation of Active Material—(1) Preparation of gland powder. The frozen glands are thawed out and ground to a coarse pulp by passing them five times through a meat grinder. The pulp is mixed with coarsely ground solid carbon dioxide and refrozen. This material is passed through a mechanical grinder (Straub), previously chilled with dry ice, first with a coarse setting of the cutting plate and again with a fine plate setting. After the solid carbon dioxide has evaporated from the powder in the cold, the ground tissue is suspended in acetone (250 ml. per 100 gm. of fresh glands) for 10 minutes and filtered by suction. This treatment is repeated with one-half the amount of acetone. The residue is then stirred with chloroform (200 ml. per 100 gm. of fresh glands) for 20 minutes and filtered by suction. The treatment is repeated with one-third the amount of chloroform. The tissue powder is rinsed with a little acetone and dried in an air stream. The material may be extracted immediately or preserved in the cold until required. It appears to retain its activity for at least several months.

(2) Hydrochloric acid extraction. The gland powder is suspended in warm (40°) 0.2 N hydrochloric acid (10 ml. per gm. of powder). The mixture is heated to 70–80° in a water bath for 5 minutes, after which the bath is removed and the extraction is continued for 2 hours. Efficient mechanical stirring is maintained throughout the entire process. The mixture is centrifuged for 45 minutes at 1500 R.P.M., and the opaque supernatant liquid is decanted. The residue is washed repeatedly on the centrifuge with small lots of distilled water until the volume of the combined supernatant liquids totals 1.5 times that of the 0.2 N hydrochloric acid used in the extraction. The extract is cleared of fat and tissue particles by filtration through a pad of filter paper pulp with the aid of a little suction. A water-clear or slightly opalescent solution is thus obtained.

(3) Fractionation with acetone. 4 volumes of acetone are stirred into the combined extract and washings (acid to Congo red). A precipitate flocculates and settles rapidly. After the mixture has stood for 2 hours in the cold, it is filtered through a Büchner funnel. The precipitate is washed with 80 per cent acid acetone (acid to Congo red) and discarded. The washings are added to the filtrate. When the filtrate is carefully neutralized to litmus with 2 N sodium hydroxide, a precipitate forms. Precipitation is completed by increasing the acetone concentration of the mixture to 86 per cent by volume. The precipitate is allowed to settle overnight in the cold, is collected at the centrifuge, washed three times with anhydrous acetone, and dried over sulfuric acid in a vacuum desiccator in the cold.

The details of one preparation are summarized in Table I, A. The

activity of the cleared extract was estimated upon suitable amounts of a lyophilized 10 ml. aliquot.   Since the material insoluble in 80 per cent acid acetone appeared to be inert (early trials showed that it was inactive at dose levels of 5 mg. of nitrogen), no estimations were made on this fraction. The material remaining in solution in neutral 86 per cent acetone was

TABLE I

Extraction of Parathyroid Powder

Fraction		Yield	Nitrogen	Nitrogen potency*	Total units
A. Typical extraction of parathyroid gland powder†					
		gm.	*per cent*	*gm.*	
HCl extract, 10 ml. aliquot		0.147	13 0	0.019	36 ± 9	41,050
86% neutral acetone ppt.		1.719	12.1	0.208	209 ± 64	43,450
Residue		0.557	1.7	0.009		
B. Summary of results of acetone fractionation of acid extracts of gland powder					
HCl extract	D3			0.365‡	28 ± 12	10,200‡
	D4			0.360‡	36 ± 9	12,200‡
	D6			0.441‡	76 ± 17	33,500‡
90% acid acetone ppt.	D1			0.028‡	173 ± 33	4,850‡
	D2			0.036‡	181 ± 36	6,500‡
	D3			0.036‡	311 ± 75	11,200‡
86% neutral acetone ppt.	D3§			0.005‡	239 ± 35	1,200‡
	D4			0.065‡	209 ± 64	13,600‡
	D6			0.053‡	560 ± 144	29,680‡
C. Fractionation of crude parathyroid preparation (Ross)					
Starting material		19.5	12.8	2.496	52 ± 15	129,800
86% neutral acetone ppt.		8 5	14.4	1.224	184 ± 47	225,200
Same, retested 30 days later					109 ± 20	133,400

* Potency in U. S. P. units per mg. of nitrogen.

† Extraction D4, 28.5 gm. of parathyroid gland powder (equivalent to 318 gm. of fresh glands); final volume of cleared extract, 600 ml.

‡ Yield in gm. of nitrogen, and total units, expressed per 100 gm. of fresh glands.

§ Bulk of active material removed in 90 per cent acid acetone precipitate.

recovered by distilling off the acetone under reduced pressure, dialyzing the aqueous solution against distilled water in the refrigerator, and lyophilizing the neutral dialyzed solution.   The residue contained a little nitrogen accompanied by some non-nitrogenous, non-dialyzable material.   In two estimations, the nitrogen potency of this fraction was found to be 129 ± 31 and 126 ± 30 units.   No considerable amount of active material is therefore lost in the residue.

Table I, B summarizes data from five preparations made in the course of developing the procedure. In each instance a large amount of inert material was precipitated from 80 per cent acid acetone solutions of the extracts. Most of the active material could be precipitated from acid solution by raising the acetone concentration to 90 per cent, but the recovery of activity was more complete if the 80 per cent acid acetone solution was first neutralized and then brought to 86 to 90 per cent of acetone. Table I shows in the estimates of total activity in the hydrochloric acid extract and in the estimates made upon the active fractions that the procedure brings about a 7- to 10-fold increase in activity with little or no loss, and that the total yields are of the order of 10,000 units per 100 gm. of fresh glands.

At a time when fresh parathyroid glands were unobtainable, we were provided with 810 gm. of a dried beef parathyroid gland powder which had been prepared as follows:[1] 10 pounds of fresh beef parathyroid glands were ground through an Enterprise grinder into 16 liters of acetone. The mixture was stirred for an hour, filtered, and stirred for an hour twice with 4 liters of acetone. The tissue was then stirred for an hour twice with 4 liters of ether, the first mixture being allowed to stand overnight. The tissue was filtered off, dried in air, and ground through a corn mill. Three lots of 90, 72, and 54 gm. of this material were extracted with dilute acid and fractionated with acetone, as described above. No highly active fractions were obtained. The indicated nitrogen potency of the best acid extract was less than 10 units, and that of the 90 per cent neutral acetone precipitates was only 30 to 40 units. These failures must be reported without an adequate explanation. The amounts of nitrogen extracted and the partition of nitrogen between the acid and neutral acetone fractions were not very different from those observed in the earlier preparations. It may be pointed out that in our procedure the defatting of the glands is less thorough and is carried out in the cold, and the final grinding is done at low temperature. It is possible that dry grinding at room temperature denatures and destroys the activity of the material

This disturbing experience led us to repeat our procedure on a small batch of 175 gm. of fresh frozen beef parathyroid glands that had been held in reserve. This was Preparation D6 (Table I, B). The results were satisfactory and were not inconsistent with those of the earlier preparations, although the estimates of potency both of the extract and of the neutral acetone fraction were higher than usual. In these estimations the rats used had somewhat lower initial levels of serum inorganic phosphorus than

were ordinarily found. The adjustment of the observed responses to the same initial level as that for the observations on the standard curve (3) was larger than usual, and it had the effect of increasing the response in every instance. The errors of the estimates indicate that they do not fall far outside of the expected range in a series of observations. It may be concluded, then, that reproducible results can be obtained if the entire procedure of preparation and extraction is followed exactly.

Through the generosity of Dr. William F. Ross, then of the Department of Biochemistry, Harvard Medical School, about 20 gm. of a crude parathyroid hormone preparation were given to us. This is the starting material for the ammonium sulfate fractionation of Ross and Wood (2). Its nitrogen potency, estimated by the dog serum calcium method (4, 5), was stated to be about 30 units. Our estimate, with the rat serum phosphorus method (3), was in fair agreement: 52 ± 15 units. 19.5 gm. of this material were dissolved in 1300 ml. of 50 per cent acid acetone (pH 2), inert material was precipitated by raising the acetone concentration to 80 per cent, and an active fraction was obtained as usual from 86 per cent neutral acetone. This precipitate was dissolved in dilute acid, forming a solution of pH 3.5, and solid ammonium sulfate was added to 0.6 saturation. The solution stood overnight in the cold. It was then centrifuged and the precipitate was suspended in water, thoroughly dialyzed against distilled water, and finally lyophilized. The results of the estimates of potency on this material are presented in Table I, C. The two estimates of potency do not differ significantly. About one-half of the nitrogen of the starting material was recovered in the active fraction, and the increase in activity was about 2-fold. The level of nitrogen potency attained in this fractionation was much less than that of the similar fractions of Table I, B. and it is about the same as that attained by Ross and Wood (2) after ammonium sulfate fractionation of their starting material. Although the recovery of total activity in the acetone fractionation is good, the results suggest that material obtained by digestion of the glands in hot acid is not as susceptible to fractionation by acetone as the product of the mild acid extraction of the gland powder.

Some General Properties of Active Fractions—The active materials obtained by the new method are light brown amorphous powders. They dissolve readily in dilute acid to form clear yellow-brown solutions. Like the preparations of Ross and Wood, they are fairly insoluble over the range, pH 4.5 to 10. The active material is soluble in 80 per cent alcohol and in 80 per cent acid acetone, but is largely insoluble in 90 per cent acid or neutral acetone. The 90 per cent acid acetone precipitates contain 13 to 14 per cent of nitrogen; the neutral acetone precipitates contain varying amounts of sodium chloride and the nitrogen contents are diminished

proportionately. The preparations gave positive biuret and negative Molisch tests.

At pH 3.5 and -5-$0°$, ammonium sulfate in concentrations greater than 0.22 saturated precipitated active material, but no concentration of activity was gained in this process and about 50 per cent of the total activity was lost. Attempts to secure more active fractions by isoelectric precipitation were only moderately successful. In one instance, 1.014 gm. of a 90 per cent acid acetone precipitate (Fraction D2, Table I, B) were dissolved in 25 ml. of water. To this solution (initial pH 2.0) 0.1 M sodium hydroxide was added slowly until maximum precipitation was obtained at pH 6.8. The mixture was centrifuged, the water-clear supernatant solution was decanted and lyophilized, and the precipitate was washed four times with anhydrous acetone and dried *in vacuo* over sulfuric acid. The

TABLE II

Isoelectric Precipitation of 90 Per Cent Acid Acetone Fraction of Parathyroid Gland Extract

Fraction	Amount	Nitrogen		Nitrogen potency	Total units
	gm.	*per cent*	*gm.*		
90% acid acetone fraction, D2	1.014	14 0	0 142	181 ± 36	25,700
pH 6.8 ppt.	0.578	15 6	0.090	162 ± 36	14,600
" 6.8 supernatant	0.299	11.3	0.034	354 ± 88	12,000

products were weighed, analyzed for nitrogen, and assayed, with the results shown in Table II. The recovery of total activity was good and the supernatant fraction, one of the most active achieved in this work, represented about 50 per cent of the total activity. Repeated isoelectric precipitation might have increased the yield of more highly active material, but, since the partition of activity was nearly equal and the more active fraction was still heterogeneous, this procedure was not considered an efficient step in the purification of the hormone.

The electrophoretic behavior óf the more active fraction from the isoelectric precipitation and of a 90 per cent acid acetone precipitate (Fraction D3, Table I, B) was studied in a Tiselius apparatus with the cell modification suggested by Longsworth. The protein boundaries were observed with the aid of the Philpot scanning device, and were photographed at suitable time intervals. For the experiments, 2 per cent solutions of the materials in acetate buffer (0.1 M, pH 3.5, ionic strength 0.1) were prepared and dialyzed against the buffer for 15 hours. The results are illustrated in Fig. 1. Both of the preparations are seen to be hetero-

A   B
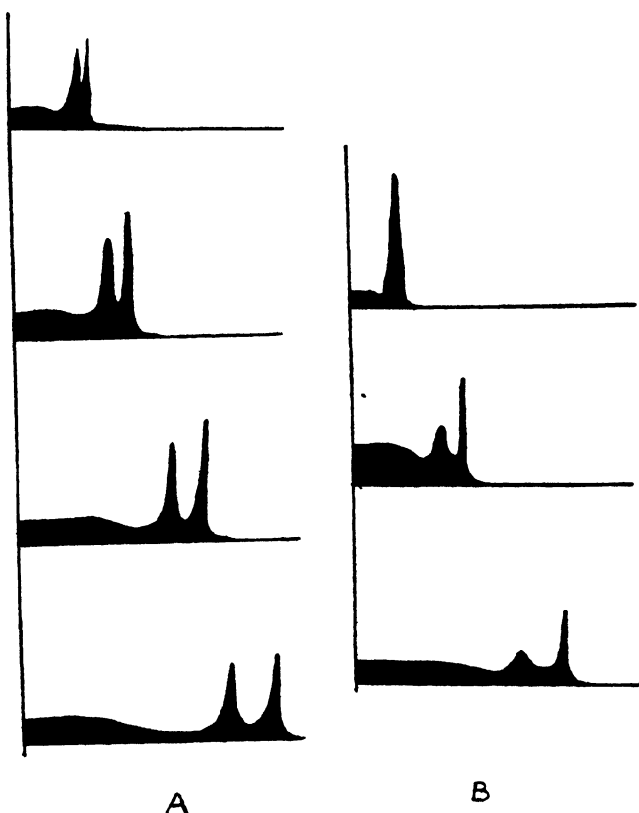
FIG. 1. Electrophoretic patterns of the ascending boundaries in two experiments with parathyroid hormone preparations. Protein concentration, 2 per cent, buffer, 0.1 M acetate, pH 3 5, 0.1 M sodium chloride, ionic strength 0 1; temperature 3°; current 3 milliamperes; voltage 30 volts. A, Fraction D3, exposures (top to bottom) at 70, 120, 180, and 270 minutes; B, fraction from supernatant solution in isoelectric precipitation (pH 6.8) exposures (top to bottom) at 0, 145, and 250 minutes.
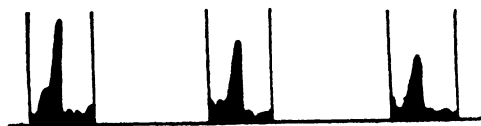
FIG. 2. Analytical ultracentrifugaton of crude parathyroid hormone preparation (method of Collip and Clark). Protein concentration, 2.4 per cent in 0.1 M NaCl, weakly alkaline (pH 8.2). 14 mm. of fluid cell. 750 R.P.S.; final rotor temperature 14°, exposures, left to right, at 20 minute intervals.

geneous, containing at least two components in about equal amount. If the activity is associated with only one of these fractions, one might

expect to find in a pure (or a homogeneous) preparation a nitrogen potency of the order of 500 to 800 units per mg. Since the preparations were not homogeneous, no attempt was made to determine the electrophoretic mobilities accurately. A rough calculation from the present data indicates that for the leading fraction $\mu = 4.8 \times 10^{-4}$ cm.^{2} volt^{-1} sec.$^{-1}$, and for the slower component, $\mu = 3.6 \times 10^{-4}$ cm.^{2} volt^{-1} sec.$^{-1}$.

The 2 per cent solution of Fraction D3 used above was also examined in a Beams air-driven analytical ultracentrifuge equipped for optical study by the Toepler schlieren method, as modified by Philpot. The solution was contained in a 14 mm. (fluid) Lucite cell in the analytical rotor. The run was carried out for 1 hour at 300 R.P.S. (23,600g) and for 1 hour at 500 R.P.S. (65,400g). During the latter period the initial protein boundary shifted slightly, owing to cell leakage, but there were no signs of any sedimenting fraction. This material does not therefore appear to contain protein of high molecular weight (240,000 or more) such as Ross and Wood (2) reported in their most active preparations.

In another experiment a 2.4 per cent solution of a preparation made by the classical method of Collip and Clark (1), with a nitrogen potency of 100, was run in the analytical centrifuge for 3 hours at 750 R.P.S. (146,000g). Fig. 2 is a tracing of photographs of the sedimentation boundaries observed during the run. Two small rapidly sedimenting components and two principal lighter components may be seen. Of the latter, the leading fraction had a sedimentation constant of 11.5 Svedberg units (molecular weight of the order of 250,000), and the lighter component had a sedimentation constant of 4.1 Svedberg units, indicating that its molecular weight lay between 60,000 and 80,000. There was also some lighter material that did not sediment during the run, so that at least five components were present in this crude preparation. The results of a partial separation of this mixture by ultracentrifugation in a quantity rotor suggested that the activity was associated with the lighter components, which is in agreement with the conclusions of Ross and Wood, drawn from a similar experiment with their material, and with the observations cited above that no component of high molecular weight is present in the material prepared by the method described in this paper.

DISCUSSION

This study began with a series of attempts to purify crude parathyroid hormone preparations made by the method of Collip and Clark (1). Ammonium sulfate, lead acetate, dilute trichloroacetic acid, and acid alcohol, alone or in combination, were neither consistent nor efficient in concentrating the active principle. In one instance, fractionation with acid acetone

yielded highly active material, but the attempt could not at first be repeated. The low yields and low and variable potency of the crude preparations made them inefficient as starting materials even for an effective method of purification.

The careful preparation at low temperatures of a dry fat-free gland powder made it possible to use milder methods of extraction which would lessen the chance of inactivating the hormone (6) and which would not alter those properties of the accompanying inert protein upon which their clean cut chemical separation from the active principle might depend. The mild extraction of the gland powder yielded a solution of high total activity, no longer susceptible of further fractionation by the method of Collip and Clark but easily fractionable, without serious losses of activity, with acetone. A direct extraction of the powder with acid acetone also yielded highly active material, but it was accompanied by a pigment most troublesome to remove, so this procedure was not adopted.

The preparations described here are similar to those of Ross and Wood (2) in respect to their protein character, the apparent absence of carbohydrate in them, their solubility at different hydrogen ion concentrations, and the order of their nitrogen potency. They differ in not appearing to contain a component of high molecular weight and in the greater yield of active substance per unit weight of fresh glands.

This material was not more thoroughly characterized because, as the electrophoresis experiments showed, it is heterogeneous, and the true properties of the hormone are therefore still in doubt. This simple method, however, improves the opportunities for purification, and it is hoped that it will contribute toward the isolation and proper characterization of the pure parathyroid hormone.

SUMMARY

A simple method of making parathyroid hormone preparations is described. It comprises (1) the preparation at low temperatures of a dry, fat-free gland powder; (2) a gentle extraction with dilute hydrochloric acid; (3) fractionation of the extract in two steps, with acid and neutral acetone. The procedure yields material of potency 200 to 300 U. S. P. units per mg. of nitrogen in quantities of 10,000 or more units per 100 gm. of fresh glands.

Some of the properties of the preparation are described. Electrophoresis shows it to be heterogeneous, containing at least two components. Ultracentrifugal analysis shows that no substance of high molecular weight is present.

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THE MECHANISM OF ACTION OF THE ANTIFATTY LIVER FACTOR OF THE PANCREAS

III A COMPARISON OF HYDROLYZED AND UNHYDROLYZED CASEIN IN THE PREVENTION OF FATTY LIVERS OF THE COMPLETELY DEPANCREATIZED DOG MAINTAINED WITH INSULIN*

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The fatty livers that appear in completely depancreatized dogs maintained with insulin and fed a high protein diet can be prevented by the administration of one of the following: (a) choline (1), (b) methionine (2), (c) fractions derived from raw pancreas (3), and (d) pancreatic juice (4). The action of the last two cannot be accounted for by their methionine and choline contents (3, 4). Evidence has also been presented in support of the view that the antifatty liver factor contained in pancreatic juice is identical with that of raw pancreas (4).

Since methionine prevents fatty infiltration in the liver of the rat by providing methyl groups for the synthesis of choline (5), it was argued (2) that the synthesis of choline from methionine is not interfered with in the dog deprived of its pancreas. Moreover, in view of the fact that the diet received by these depancreatized dogs while developing fatty livers is not deficient in methionine (each dog received 500 gm. of lean meat per day) it was postulated that the antifatty liver factor contained in the external secretion of the pancreas and in fractions isolated from raw pancreas is like a proteolytic enzyme which is necessary for ingested proteins to exert their full lipotropic effects (6).

Further evidence in support of this hypothesis is presented in this report. It is demonstrated here that purified casein, the feeding of which resulted in the development of fatty livers in the completely depancreatized dog maintained with insulin, can be made to prevent fatty livers by hydrolyzing it before feeding.

EXPERIMENTAL

Preparation of Casein Hydrolysate[1]—1 kilo of Labco casein (vitamin-free, choline-free) was added with thorough shaking to 5 liters of cold 5 N H_2SO_4

and the mixture autoclaved for 16 hours at 15 pounds pressure.   While the mixture was still hot, 200 gm. of animal charcoal (Nuchar) were added, and the mixture shaken for 5 minutes and filtered through a Büchner funnel.   The residue was then washed with a liter of boiling water.

The filtrates were combined and heated to 80°.   Slaked lime was added to the mixture to bring it to pH 4.5; it was then filtered through a Büchner funnel with suction.   The residue was mixed once with hot water (80°) and the resulting mixture filtered.   The combined filtrates were heated to 80°, powdered barium hydroxide added to adjust the pH to 7.2 (glass electrode), and the mixture filtered while still hot.   The barium-free filtrate obtained was dehydrated by lyophilizing.   The dry powder so

TABLE I

Composition of Diets

Diet	Protein source	Meat	Labco casein*	Casein hydrolysate	Sucrose	Galen B†	Salt mixture‡	Bone ash	Sardilene†	Cellu flour	Lard	dl-Tryptophane	dl-Methionne
		gm	gm	gm	gm	cc	gm	gm	cc	gm.	gm	gm	gm
A	80 gm. casein		80		100	5	2	10	3		10		
B	80 Hydrolyzed casein			80	100	5	2	10	3	20	10	0 8	
C	Meat, 80 gm. "	500	80		100	5	2	10	3				
D	" 20 " hydro-lyzed casein	500		20	100	5	2	10	3			0 2	
E	Meat, 0.6 gm. methio-nine	500			100	5	2	10	3				0.6

* Stated to be vitamin-free, no choline could be isolated from it.
† For the vitamin content see (6).
‡ After Cowgill (7).

procured was quickly divided into portions sufficient for one meal, each one transferred to a tightly stoppered bottle which was kept at 6° until used. Since tryptophane of casein is destroyed by acid hydrolysis, this amino acid (*dl* form) in an amount equivalent to 1 per cent of the casein hydrolysate was added to each dietary mixture fed.

Preparation of Animals—The depancreatized dogs used in this study were prepared as previously described (6).   When they had regained their appetites and were in good condition, the raw pancreas was omitted from their diets and the diets described in Table I were then fed for the next 10 to 20 weeks.   Each dog received one-half of the specified amounts at 8 a.m. and the second half at 4 p.m.   8 units of insulin were injected after each meal.

At the end of the test period the dogs were anesthetized with nembutal, and their livers removed and analyzed for fatty acids in a manner previously described (8).

Results

In view of the effects of free methionine (2), the question arose as to

TABLE II

Effect of Very High Protein Intake on Development of Fatty Livers by Completely Depancreatized Dogs Maintained with Insulin

The dogs were fed Diet C daily for 20 weeks.

Dog No	Body weight			Liver	
	At start	At starting Diet C	Final	Weight	Total fatty acids
	kg.	*kg*	*kg.*	*gm.*	*per cent wet weight*
D567	12.5	11.2	9.7	855	22.9
D568	9.5	9.7	9.5	700	16.8
D569	14.2	13.0	11.9	432	19.7
D582	11.8	10.4	10.0	545	3.7

TABLE III

Comparison of Hydrolyzed and Unhydrolyzed Casein on Production of Fatty Livers in Completely Depancreatized Dog Maintained with Insulin

Dog No	Daily diet	Body weight			Wks. fed	Liver	
		At start	At starting Diet A or B	Final		Weight	Total fatty acids
		kg.	*kg.*	*kg.*		*gm.*	*per cent wet weight*
D339	A	11.1	10 3	9.7	12	452	16.4
D380	"	11.6	10.9	7.6	20	440	17.5
D398	"	14.5	14.3	8.2	16	397	16.6
D405	"	16.5	17.5	14.2	17	550	5.9
D407	"	12.8	12.5	7.5	16	680	19.3
D586	B	11.8	10.6	9 5	10	500	1.9
D590	"	11.2	10.5	8 7	16	600	1.6
D591	"	12.5	11.0	8.8	16	465	2.0

whether the fatty livers observed in depancreatized dogs fed a lean meat diet could be overcome by increasing their protein intake. Four dogs (Nos. D567, D568, D569, and D582, Table II) were therefore fed daily for 20 weeks 500 gm. of lean meat and 80 gm. of casein. Despite the fact that this amount of protein contained 7 gm. of methionine, fatty livers were found in three of these four dogs. This confirms an earlier observation that the daily ingestion of 1000 gm. of lean meat did not prevent the

infiltration of large amounts of fat in the liver of a depancreatized dog (9).

In Table III the effects of feeding casein and hydrolyzed casein upon the development of fatty livers are compared.   There can be little doubt from these results that the failure of 80 gm. of casein to prevent fatty livers is due to the inability of the depancreatized dog to release an effective amount of methionine from this casein.

The results shown in Table IV demonstrate that the methionine content of 20 gm. of casein can fully account for the lipotropic activity of this amount of hydrolyzed casein.   The dogs in this experiment were fed 500 gm. of lean meat daily.   In addition, the first five dogs received daily 20 gm. of hydrolyzed casein, whereas the last three dogs received daily 0.6

TABLE IV

Showing that Antifatty Liver Action of 20 gm of Hydrolyzed Casein Can Be Accounted for by Its Methionine Content

Dog No	Daily diet (20 wks)	Body weight			Liver	
		At start	At starting Diet D or E	Final	Weight	Total fatty acids
		kg	*kg*	*kg*	*gm.*	*per cent wet weight*
D576	D	14.0	12.8	11.2	503	6.6
D578	"	10.5	10.2	10.9	380	2.2
D579	"	7.5	6.6	8.7	400	3.1
D571	"	10.1	9.5	9.3	422	4.9
D580	"	14.0	13.5	12 0	545	3.7
D599	E*	9.2	7.0	7.4	417	4.8
D601	" ·	12.0	10 6	8 9	387	4.7
D607	"	9.8	9.1	10.0	315	2.6

* Each animal received a total of 84 gm. of methionine during the 20 week period of observation.

gm. of free methionine.   Both types of supplementary treatment completely prevented the development of fatty livers.

DISCUSSION

The hypothesis formulated in an earlier communication to explain the development of fatty livers in completely depancreatized dogs maintained with insulin is fully supported by the experimental evidence presented in the present investigation.   The pronounced difference in the antifatty liver action of hydrolyzed and unhydrolyzed casein and the demonstration that the antifatty liver effect of 20 gm. of casein can be accounted for by its methionine content justify the conclusion that in the gastrointestinal tract of the completely depancreatized dog maintained with insulin there is an

interference in the mechanism whereby the methionine of protein is made available for lipotropic purposes. This defect, so far as the fatty liver is concerned, can be overcome by the feeding of choline or of its precursor, methionine, or of fractions isolated from raw pancreas. Although preliminary investigations in this laboratory and in Best's laboratory (10) have shown that trypsin and chymotrypsin are present in purified pancreas fractions, the question whether trypsin and antifatty liver activity are identical must be left open for the present.

SUMMARY

1. In contrast to unhydrolyzed protein which, even when fed in very large amounts, fails to stop the development of fatty livers, *hydrolyzed* casein readily prevents fatty livers in completely depancreatized dogs maintained with insulin.

2. The antifatty liver action of 20 gm. of *hydrolyzed* casein in this animal preparation can be accounted for by its methionine content.

3. The evidence presented here supports the view that a defect responsible for the development of fatty livers in the completely depancreatized dog maintained with insulin is in the mechanism by which the methionine of ingested protein is made available for lipotropic purposes.

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THE RAPID ESTIMATION OF TYROTHRICIN IN FERMENTATION LIQUORS

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The isolation of tyrothricin from fermentation liquors by the method of Dubos and Hotchkiss (3) is, from a production standpoint, the most satisfactory method of determining yields. The time and labor involved in the isolation, however, make the method unsuitable for routine use when dealing with many samples. The usual serial dilution technique for determining the bacteriostatic concentration against a test organism will give a good estimate of the amount of tyrothricin in an unknown solution if the method is properly standardized and if a sufficient number of dilutions are used. Here, also, the time and labor involved preclude routine use.

Dimick (2) developed a method based on the hemolytic properties of tyrothricin "by means of which it is possible to determine as little as 100 mg. of tyrothricin per liter of media, with an accuracy of about 5 per cent." This method is satisfactory for routine work, but it is inconvenient in several respects, in particular because of the need for establishing a standard curve for every lot of blood used, the narrow range of tyrothricin permissible in the alcoholic solution assayed, and the necessity of having a convenient source of blood.

Hotchkiss (8) pointed out that Dimick's method essentially measures the tyrocidine-like components of tyrothricin, since under the conditions of the determination gramicidin would have no hemolytic effect, and Hoogerheide (5) criticized the method on the grounds that one may not assume that tyrocidine and gramicidin are present in constant proportion during the course of the entire growth cycle. However, a good correlation was obtained between the results of the hemolytic assay and isolated tyrothricin under a wide variety of culture conditions (2, 9), an observation that lends support to the view that the ratio of gramicidin to tyrocidine is remarkably constant under a wide variety of conditions (8).

In order to expedite a study of the factors which influence tyrothricin production, an attempt was made to develop a chemical method of analysis. If, as the results of Dimick indicate, the proportion of gramicidin and tyrocidine is constant under a wide range of culture conditions, then the

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determination of any amino acid present in either or both components of tyrothricin should give a satisfactory measure of the total. Since it has been established by Hotchkiss and others (7, 1, 4) that tryptophane is a component of both gramicidin and tyrocidine, accounting for 40 per cent of the nitrogen of the former and 15.4 per cent of the latter, a colorimetric method for its determination in tyrothricin was chosen as a means of assaying tyrothricin-containing liquors.

The tryptophane method of Horn and Jones (6) was applied to alcoholic extracts of material precipitated from fermentation liquors. The color produced correlated with the amount of tyrothricin isolated from the same liquors and could be used as a rapid, routine method for estimation of tyrothricin.

<center>EXPERIMENTAL</center>

Tyrothricin was produced in shake flask cultures of *Bacillus brevis* with the medium of Stokes and Woodward (10), and isolated by previously . described methods (3). As a check on the identity of the product, crystalline gramicidin and tyrocidine were isolated and identified by physical and chemical tests. In a mouse protection test, 1.35 γ of the isolated tyrothricin protected 50 per cent of the mice used against 12,600 minimum fatal dose of type I pneumococcus.[1] When assayed in serum albumin peptone medium by the method of Dubos and Porter,[2] the isolated material inhibited growth of a Group D hemolytic streptococcus (Lancefield strain[3] ($H_{69}D_5$) at a concentration between 0.20 and 0.25 γ per ml. The above activities are comparable to those obtained with tyrothricin samples prepared in other laboratories. A composite sample of six lots of tyrothricin having the listed properties was used as a standard.

Assay Procedure—Weighed samples of the standard were dissolved in alcohol[4] to give solutions of the desired concentration. The alcoholic solutions are stable and can be held indefinitely. The color is developed by adding 0.5 ml. of 5 per cent *p*-dimethylaminobenzaldehyde (prepared fresh daily) in concentrated HCl and 0.5 ml. of alcohol to 5.0 ml. of concentrated HCl. This is followed by 1 ml. of the tyrothricin solution. If tyrothricin is present, a pink color develops almost at once. After 5 minutes, 1 drop of 0.2 per cent aqueous sodium nitrite is added and a blue color appears in a few seconds. The color density is read after 15 minutes in a Klett-Summerson photoelectric colorimeter with a blue filter (400 to

465 mμ). A tube containing 1.5 ml. of alcohol, 5 ml. of concentrated HCl, 0.5 ml. of the 5 per cent p-dimethylaminobenzaldehyde solution, and 1 drop of 0.2 per cent sodium nitrite is used as a blank.

Table I shows the color densities obtained from different amounts of the standard tyrothricin sample. The densities are averages of three separate determinations. The ratio of tyrothricin to color density (K) is almost constant with a maximum deviation from the average K of 5.3 per cent and an average deviation of 1.7 per cent. It is obvious that the color developed is directly proportional to the amount of tyrothricin over the range tested.

TABLE I

Color Densities from Known Amounts of Tyrothricin

Tyrothricin	Color density*	Spread†	K, tyrothricin to density	Deviation from mean K
γ				*per cent*
25	11.7	7	2.14	5.3
50	22.5	9	2.22	1.8
75	32	12	2.34	3.5
100	44.7	5	2.24	0.9
200	87	12	2.30	1.8
300	131	7	2.29	1.3
400	178	5	2.25	0.4
500	217.3	4	2.20	2.7
600	262.5	3	2 29	1.3
700	307.3	7	2 28	0 9
800	351	0	2 28	0.9
900	397	3	2.27	0.4
1000	441	0	2.27	0.4

* Average of three determinations; Klett-Summerson photoelectric colorimeter.
† Difference between maximum and minimum color value.

The maximum spread between the high and low color reading for a given amount of tyrothricin is 12 color units with an average spread of 6 units. In terms of tyrothricin, this represents 13.5 γ to 27 γ, the minimum uncertainty in any single determination.

Assay of Fermentation Liquors—To assay a culture medium, 5 to 10 ml. of a representative sample are accurately measured into a centrifuge tube. The pH of the liquor is adjusted to between 4.0 and 4.5 by addition of a predetermined quantity of hydrochloric acid. The cells and precipitated matter are centrifuged down and the clear supernatant liquor is decanted. Exactly 5 ml. of alcohol are added to the tube, the sediment is resuspended, the tube is shaken for 10 minutes, allowed to stand for 20 minutes, and

recentrifuged. 1 ml. of the clear alcoholic extract is used for the color development. This quantity is equivalent to 1 to 2 ml. of the fermentation liquor, depending on the size of the initial sample. The range can be increased if necessary by varying the size of the initial sample or the volume of alcohol used for extraction.

Routinely 1 ml. of a solution containing 200 γ of the standard is run along with each set of unknowns. This quantity will give a color reading of 85 ± 4 color units. The quantity of tyrothricin in the unknown is calculated by multiplying the ratio of the unknown color value to the standard color value by 200.
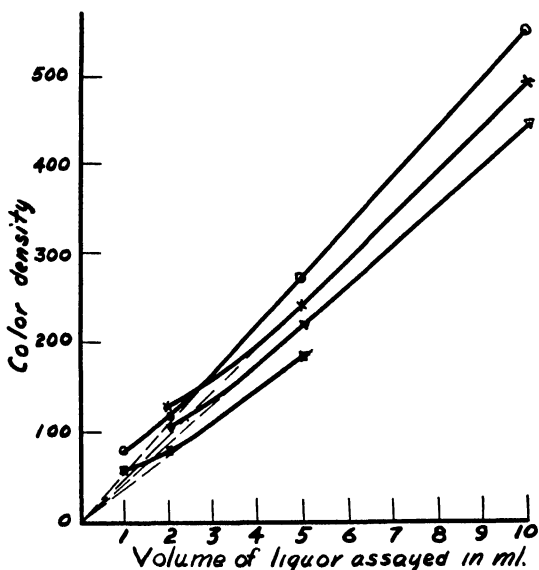
Fig. 1. The relation between color density in scale units of Klett-Summerson photoelectric colorimeter, and volume of culture liquor assayed.

If oils have been used as antifoaming agents in the course of the fermentation, the final color solution will be cloudy. In such cases, extraction of the colored solution with carbon tetrachloride followed by centrifuging will remove the cloudiness without affecting the color density.

The accuracy and reliability of the method when applied to fermentation liquors were assessed in several ways, by analysis of different volumes of the same liquor, by isolation of tyrothricin from assayed liquors, and by assays of liquors to which known quantities of pure tyrothricin were added. Fig. 1 gives the results of four experiments in which different volumes of the same liquor were assayed. When samples greater than 3 or 4 ml. were used, the color densities fall on straight lines that intersect

at the zero point. Smaller samples gave densities that were relatively high and the amount of tyrothricin calculated from these values will be proportionately high. The cause of the abnormal results with small samples has not been determined but the difficulty is avoided by using a sample sufficiently large to give a color reading of at least 150. On several occasions, when large amounts of tyrothricin were present, the initial alco-

TABLE II

Comparison of Assay Values and Isolated Tyrothricin

Total tyrothricin by color assay	Isolated tyrothricin*	Recovery
gm.	*gm.*	*per cent*
4.82	3.90	81
0.65	0.58	89
2.33	1.92	82
4.44	3.56	80
3.10	2.40	78
3 45	3.20	93

* The weight of the final purified product having a bacteriostatic potency equal to the standard employed.

TABLE III

Recovery of Tyrothricin Added to Fermentation Liquor

	Tyrothricin			Recovery†	Deviation‡
Original culture	Amount added	Total calculated	Total found*		
γ per ml.	*γ per ml.*	*γ per ml.*	*γ per ml.*	*per cent*	*per cent*
	0		186		
186	25	211	203	96	5.0
186	50	236	232	98	1.0
186	100	286	284	99	4.5
186	200	386	400	103	3.6

* Average of three determinations.
† (Found)/(calculated) × 100.
‡ Maximum deviation from mean of three determinations.

holic extract was decanted and the sediment reextracted with a fresh portion of alcohol. The second extract has never shown more than a trace of color on assay, thus eliminating the possibility that the tyrothricin was not completely extracted. Occasionally, when the tyrothricin level is negligible, especially very early in the fermentation, a visible yellow-green to green color instead of the usual pure blue develops in the solution. The color density is not significant when this occurs.

Many experiments were run in which tyrothricin was isolated from

different culture media after assay. On the average about 80 per cent of the assayed value has been recovered by isolation. In one experiment the recovery was as low as 52 per cent and in one over 100 per cent was recovered. In general, low recoveries were usually associated with mishaps in the isolation procedure or with runs in which the total amount of tyrothricin was less than 100 mg. In no case has tyrothricin been isolated from liquors that gave no tryptophane color in the assay. Table II gives the results of the last six isolations made.

As a check on the accuracy and sensitivity of the method covering both the extraction and color development, experiments were run in which varying amounts of tyrothricin were added to a completed fermentation liquor and the culture was then assayed as described. Table III gives the results of one experiment. The determinations were run on triplicate samples. The data show that a difference of 25 γ per ml. can be detected with an accuracy of 5 per cent or better.

<center>DISCUSSION</center>

Several thousand fermentation liquors have been assayed by the method described. One technician can do as many as forty or fifty determinations in the course of a morning's work. No difficulties have been encountered when a minimum of 50 to 100 γ of tyrothricin per ml. of medium has been present. The method has been applied in studying the optimum conditions for tyrothricin production and the effect of various constituents of the medium on the yield. It is particularly useful in following the course of a fermentation and determining the time of maximum yield. Differences of as little as 25 γ per ml. can be detected with an accuracy of about 5 per cent.

The method presented is not specific for tyrothricin but is dependent upon the tryptophane moiety of the complex, and therefore is open to at least two serious criticisms: (a) The proportion of gramicidin to tyrocidine under changed conditions of fermentation or with different strains of organisms might vary; however, both Dimick's results (2, 9) and our own indicate that such a variation has not occurred in the range of conditions tested. (b) It is also possible that non-antibiotic substances containing tryptophane soluble in alcohol and insoluble in water might be formed in the various cultures, thus leading to high results.

The fact that only 80 per cent of the tyrothricin determined by the described method is actually isolated from the culture media can be explained in two ways; either interfering substances contribute to the color intensity or the isolation procedure is not quantitative. Considering the number of steps involved in the isolation, one might well expect an appreciable loss of active material. It is of interest that the amount of

tyrothricin isolated by Dimick from various fermentation liquors also was about 80 per cent of that found by his assay method (2).

SUMMARY

A rapid colorimetric method for the determination of tyrothricin in culture media based on a tryptophane assay has been described. The method can determine quantities as small as 25 γ per ml. with an accuracy of 5 per cent or better.

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GLYCINE AND ALANINE CONCENTRATIONS OF BODY FLUIDS; EXPERIMENTAL MODIFICATION

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The determination of individual amino acids in body fluids under pathological and experimental conditions should permit extension of information farther than is possible with procedures that measure amino acids collectively. We are reporting some observations upon experimentally produced changes in the concentrations of glycine and alanine, as measured by chemical methods (1, 2) which are specific to the uncombined forms of these amino acids.

The ingestion of 25 to 32 gm. of glycine by human subjects resulted in rises of the plasma concentration of glycine nitrogen from the normal of 0.28 to 0.37 mg. per cent to values of 2.9 to 7.7 mg. per cent, the maximum being attained in about an hour. Glycine entered the erythrocytes slowly, the concentration remaining below that of plasma for more than 4 hours (Table I). After the glycine nitrogen of plasma had been held at levels of 3 to 5 mg. per cent for 4 to 5 hours by the ingestion of several portions of glycine totaling 30 and 35 gm., the glycine nitrogen concentrations of the spinal fluid were 0.065 and 0.07 mg. per cent, compared with five normal values of 0.022 to 0.042 mg. per cent, averaging 0.031 mg. per cent. The ingestion of 25 gm. of glycine increased the conjugated α-amino nitrogen of tungstic acid filtrates of plasma, only a small part of the increase being due to glycine. The bound glycine extractable by ethyl acetate from acidified tungstic acid filtrates of plasma (which would include hippuric acid) was not significantly increased. The ingestion of 5 and 6 gm. of sodium benzoate led to a decrease of the free plasma glycine, a greater reduction of the plasma α-amino acids, and an increase of 0.4 to 0.7 mg. per cent in the conjugated diffusible glycine nitrogen of plasma, presumably due to hippuric acid. Table II illustrates one of these experiments.

The ethyl acetate-extractable, conjugated amino acid nitrogen of urine was taken by Henriques and Sørensen (3) as a measure of hippuric acid excretion. In human urine they found about half of the conjugated amino nitrogen to be extractable. We have partitioned the conjugates by the extraction of acidified urine, employing three extractions of 1 volume each, which was sufficient to extract 99 per cent of the hippuric acid from the aqueous solution. Whereas glycine made up only 6 to 17 per cent of the

free α-amino acid nitrogen of urine, both fractions of the conjugates were rich in glycine (Table III). The extractable portion included substantial quantities of other amino acid conjugates in addition to hippuric acid.

Ingestion of 25 gm. of *dl*-alanine gave results analogous to those for glycine in regard to sluggish distribution between plasma and erythrocytes and scarcely appreciable entrance into the cerebrospinal fluid (Table

TABLE I

Amino Acid Changes of Plasma and Erythrocytes after Ingestion of Glycine

25 gm. of glycine were ingested at zero time in Experiments W14 and Z10, 32 gm. in Experiment U40; the data are expressed in mg per cent

Experiment No.		Plasma					Erythrocyte glycine N
		Glycine N		Free alanine N	α-Amino N		
		Free	Bound		Free	Bound	
W14	Pretest	0.30	0.07		4.40	1.4	
	65 min.	2.9	0.2		7.39	2.8	0.61
	180 min.	1.51	0.28		6.01	1.6	0.92
Z10	Pretest	0.37	0.06	0.60	4.66	1.1	
	60 min.	4.2	0.5	1.04	8 86	2 9	
	180 min.	2.3	0.2	0.87	7.29	1 2	
U40	Pretest	0.32	0.07	0.57	4.92	0.1	0.46
	50 min.	7.7	0.1	1.28	12.61	1.6	1.15
	155 min.	4.0	0.7	1.12	9 26	1 1	1 72

TABLE II

Effect of Ingestion of 6 Gm. of Sodium Benzoate upon Plasma Amino Acids

Experiment	Plasma concentration		
	Pretest	90 min	180 min.
	mg per cent	*mg per cent*	*mg. per cent*
Free α-amino N .	4.58	4.18	4.51
Bound diffusible α-amino N	0 41	0.54	0.64
Free glycine N	0 39	0.28	0.30
Bound diffusible glycine N	0.10	0.49	0 49

IV). The ingestion of either glycine or alanine produced distinct increases of the plasma concentrations of the other amino acid (Tables I and IV). Such effects make inexact the assumption that elevations of the amino acid nitrogen measure the increases in concentration of a single amino acid administered alone.

When alanine and glycine were administered as 100 gm. of gelatin (equivalent to 25.5 gm. of glycine and 8.7 gm. of alanine (4)), glycine

represented 19 to 25 per cent of the increase of α-amino acid nitrogen, while alanine made up 17 to 19 per cent of the increase (Table V), indicating a

TABLE III

Contribution of Glycine to Free and Conjugated Amino Acids of Human Urine

Urine U33 was a pool of 24 hour samples from three normal persons. The other urines were collected from 10 p m. to 8 a.m , no food being taken after 6 p.m. Urine D27 represents two such 10 hour periods, Urine C27 three such periods.

Urine No	Free			Total extractable			Bound non-extractable		
	α-Amino N	Glycine N	$\frac{\alpha\text{-Amino N}}{\text{Glycine N}} \times 100$	α-Amino N	Glycine N	$\frac{\alpha\text{-Amino N}}{\text{Glycine N}} \times 100$	α-Amino N	Glycine N	$\frac{\alpha\text{-Amino N}}{\text{Glycine N}} \times 100$
	mg per hr.	*mg per hr*		*mg per hr*	*mg. per hr*		*mg per hr.*	*mg per hr.*	
C27	5.58	0.85	15 2	3.45	3.2	93	4.5	2.3	50
D27	4 54	0 28	6 2	4.39	1.44	33	3 4		
F20	5 73	0.56	9.8	2.4	0 36	15		1.6	
J57	7.05	0.44*	6 2*						
U33	4.29	0 74	17	2.25	0.95	42	4 26	1.46	34
A30†	25.9	1 74	6 4	3 67	2.2	60	9.4	2.1	22

* These values are for alanine N instead of glycine N.

† Hepatic cirrhosis with ascites; bromosulfalein retention (5 mg. per kilo dose) 48 per cent after 1 hour

TABLE IV

Effect of Ingestion of dl-Alanine

Experiment No		Alanine N	Plasma glycine N	Free α-Amino N	Erythrocyte alanine N
		mg per cent	*mg per cent*	*mg. per cent*	*mg. per cent*
T49*	Pretest	0.74	0 30	4.73	0.55
	50 min	5.03	0.45	9.32	1.44
	120 min	5.64	0.37	11.0	2.41
J57†	Pretest	1 0	0.31	4 36	0 85
	90 min	6 4	0.44		1.8
	180 min	5.0	0.39		2.0
	255 min.	5.3	0.35	7.79	2.2

* 25 gm. of *dl*-alanine taken at zero time.

† 15 gm. of *dl*-alanine taken at zero time, 5 gm. at 90 minutes, 5 gm. at 180 minutes. At 270 minutes the spinal fluid alanine nitrogen was 0.34 mg. per cent, compared with six normal values ranging from 0.12 to 0.33 and averaging 0.21 mg. per cent.

more rapid disposal of glycine than of alanine under these conditions. Evidence was sought unsuccessfully for increases of glycine and alanine peptides.

EXPERIMENTAL

The various preparations were ingested in aqueous suspension by normal subjects who had not eaten for 14 hours. Deproteinization of plasma and cells from heparinized venous blood was accomplished by tungstic acid (5, 6). 14 to 20 ml. of cerebrospinal fluid were collected by puncture at the fourth lumbar interspace, and a protein-free filtrate was prepared by dialysis for 7 hours at 5°. Free α-amino nitrogen was determined by the manometric ninhydrin procedure at pH 2.5 (7, 8) and total α-amino nitrogen likewise, after acid hydrolysis (6). Glycine and alanine were determined by the methods of Alexander, Landwehr, and Seligman (1) and Alexander and Seligman (2). Glutathione responded to the glycine determination to the extent of 0.0004 mg. of glycine nitrogen per mg. of glutathione. Hence free glycine determinations upon tungstic acid filtrates

TABLE V

Effect of Gelatin Ingestion

117 gm. of Knox gelatin (100 gm. of protein) ingested. The values are in mg per cent

Experiment No		Glycine		Alanine		α-Amino N		$\dfrac{\Delta \text{ Glycine N}}{\Delta \alpha\text{- amino N}}$	$\dfrac{\Delta \text{ alanine N}}{\Delta \alpha\text{-amino N}}$
		Free	Bound	Free	Bound	Free	Bound		
1	Pretest	0.32	0.1	0.50	0.2	4.68	0.8		
	90 min.	1.64	0.1	1.49	0.5	9 97	0 8	0 25	0 19
	210 min.	1.50	0.1	1.37	0.4	9 74	1 5	0 23	0.17
2	Pretest	0.31	0,1	0.51	0 0	3.93	0.4		
	120 min.	1.59	0.04	1.7	0.1	10.1	0.8	0 21	0.19
	240 min.	0.96	0.2	1.12	0 1	7.36	0.7	0.19	0.17

of red cells were probably subject to positive errors of about 0.03 mg. per cent of glycine nitrogen. Sodium hippurate (Eastman) yielded about 2 per cent of its glycine nitrogen under the conditions of the determination. *Bound* α-amino nitrogen and *bound* glycine were determined after acid hydrolysis of the tungstic acid filtrates, and *bound diffusible* α-amino and glycine nitrogen after hydrolysis of dialysates (9) of tungstic acid filtrates. All urine analyses were conducted upon dialysates.

DISCUSSION

The question as to whether the interval employed was sufficient to produce a response of the alanine and glycine of the cerebrospinal fluid to the elevation of the plasma levels can be answered only by comparison with the behavior of other solutes under similar circumstances. Merritt and Fremont-Smith (10) and Munch-Petersen (11) have shown that, when a

transient hyperglycemia is produced by feeding or injecting glucose, the glucose of the lumbar spinal fluid rises within an hour or 2. The responses of alanine and glycine were relatively sluggish.

The manometric ninhydrin procedure showed 1.04 to 1.43 mg. per cent of α-amino nitrogen (average 1.23 mg. per cent, six analyses) in cerebrospinal fluid. Weichmann and Dominicke (12) reported a normal range of 1.2 to 2.0 mg. per cent by the Folin colorimetric method. In two cases of liver disease with elevated plasma amino acid nitrogen, these investigators observed cerebrospinal fluid levels of 3.2 to 6.5 mg. per cent. Harris (13) has found the glutamine concentrations of cerebrospinal fluid to be similar to those of plasma, which would mean that half or more of the spinal fluid α-amino nitrogen is due to glutamine.

The large participation of the glycine in the amino acid conjugates of urine, other than hippuric acid, suggests that these conjugates are not simply a random collection of peptides wasted in the course of protein metabolism but may have a different significance. Dent has reported in a preliminary communication (14) that glycine and alanine make up a major part of the amino acids of normal human urine. He observed a peptide which was probably serylglycylglycine in the urine of patients with hepatic necrosis or the Fanconi syndrome.

SUMMARY

1. The concentrations of glycine and alanine of erythrocytes approached very gradually over several hours the levels of plasma when the latter was elevated by the ingestion of pure amino acids by human subjects.

2. Cerebrospinal fluid levels of these two amino acids were about one-tenth (glycine) and one-third (alanine) the plasma levels. When the plasma levels were elevated for several hours by ingestion of amino acids, the cerebrospinal fluid levels rose only very slightly.

3. Glycine ingestion appeared to increase the conjugated amino acid nitrogen of tungstic acid filtrates of plasma.

4. The ingestion of sodium benzoate increased the bound diffusible glycine of plasma five or ten times. The free glycine and free α-amino nitrogen were decreased.

5. The ingestion of either glycine or dl-alanine resulted in an elevation of the plasma level of the other amino acid.

6. When glycine and alanine were ingested as gelatin, alanine made up a disproportionately large part of the increase in plasma amino acids.

7. Glycine made up a small part of the free amino acid nitrogen of normal urine, but a larger part of the conjugated amino acid nitrogen, even after hippuric acid was removed.

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THE QUANTITATIVE DETERMINATION OF ASCORBIC ACID IN SMALL AMOUNTS OF WHITE BLOOD CELLS AND PLATELETS

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A knowledge of the concentration of ascorbic acid in the white blood cells and blood platelets has been shown to be of value as an addition to blood plasma analysis in evaluating nutritional status, particularly at low levels of ascorbic acid intake (1–5). Furthermore, since it is probably more convenient to obtain specimens of white blood cells than of any other living cells of the body, it seems likely that analysis of these cells will become of increasing value in studying the physiology of ascorbic acid and other nutritive essentials in body fluids and tissues. Stephens and Hawley (6) determined the ascorbic acid content of white blood cells by an analysis of the buffy layer removed from 50 ml. blood specimens. This layer contains both white cells and blood platelets. It was later shown by Butler and Cushman (1) that the concentration of ascorbic acid is nearly the same in both of these fractions. (Throughout this communication the white cells plus blood platelets will be referred to collectively as "white cells.") Butler and Cushman (1) simplified the collection of the white cells by centrifuging oxalated blood in special tubes with a central narrowed portion which converted the buffy coat into a well defined accessible column of cells. The required amount of blood, 15 ml., was subsequently reduced by Lubschez (7) to 4 or 5 ml.

By a procedure which will be described below, it has proved feasible to isolate the white cells from as little as 0.1 ml. of blood and to analyze them for ascorbic acid. Blood from the finger tip is diluted with isotonic potassium oxalate and centrifuged slowly to precipitate the red cells. The white cells, which settle more slowly, remain in the supernatant and are centrifuged in a second tube and analyzed for both ascorbic acid and acid-insoluble phosphate. The measurement of this nearly constant phosphate fraction permits calculation of the amount of white cells present without the necessity of weighing the rather minute sample. The procedure is more rapid than with larger quantities of blood and venipuncture is not required. Two persons can collect and analyze 50 to 75 samples in 2 days.

Method

Reagents and Equipment—

1. Beckman spectrophotometer fitted with a special diaphragm and cuvettes to permit the use of 0.05 ml. fluid volumes (8). (Diaphragm and cuvettes obtained from the Pyrocell Manufacturing Company, 207 East 84th Street, New York 28.)

2. 6 × 50 mm. serological tubes, *e.g.* Kimble, No. 45060. Square racks made of ⅜ inch wire mesh are convenient for handling up to 100 tubes.

3. Pipette for transferring blood (9), 5 to 6 mm. in diameter with a short tip narrowed to 1 mm. in outside diameter and with the opening at least 0.5 mm. in diameter. A coarse constriction placed at 0.1 to 0.12 ml. volume aids in preventing the accidental sucking of blood too far into the pipette. This pipette, although uncalibrated, is similar in construction to the quantitative constriction pipettes listed below.

The inside is paraffined by heating the pipette, sucking up molten paraffin, blowing it out, and, while still blowing, cooling with water. This leaves a thin film of paraffin effective in delaying blood clotting without unduly constricting the tip.

4. Footed stirring rod made of glass or stainless steel wire. The shaft is 1 to 1.5 mm. in diameter and 75 mm. long; the foot is 2.5 to 3 mm. in diameter and is flattened.

5. Pasteur pipette of 1 to 1.5 ml. capacity with bent tip for transferring white cell suspension.

6. Constriction pipettes, 10, 30, 40, 50, and 200 c.mm. (9).

7. Device for removing the supernatant from white blood cells. A small glass tube with a bent narrow tip not over 0.5 mm. in outer diameter is connected with rubber tubes, through a 20 to 50 ml. bottle, to the mouth.

8. 1.6 per cent potassium oxalate. This is preserved at 4° to prevent the growth of microorganisms and must be centrifuged just before use to remove possible traces of suspended material.

9. 5 per cent trichloroacetic acid.

10. 2 per cent dinitrophenylhydrazine, 0.25 per cent thiourea, 0.03 per cent $CuSO_4 \cdot 5H_2O$ in 9 N H_2SO_4. This reagent is stable for at least a week at 4°, but should be centrifuged before use unless crystal-clear. It is prepared from the stable solution of 2.2 per cent dinitrophenylhydrazine in 10 N H_2SO_4 by the addition of 5 volumes per cent of 5 per cent thiourea and 5 volumes per cent of 0.6 per cent $CuSO_4 \cdot 5H_2O$.

11. 65 per cent H_2SO_4 (70 ml. of concentrated H_2SO_4 plus 30 ml. of H_2O).

12. 6.5 N sulfuric acid.

13. 10 per cent perchloric acid.

14. Phosphate reagent. In 45 ml. of water is dissolved 0.3 gm. of a powder consisting of 5 per cent Na_2SO_3, 94.3 per cent $NaHSO_3$, and 0.7 per cent 1,4-aminonaphtholsulfonic acid. To this are added 5 ml. of 2.5 per cent ammonium molybdate in water.

Isolation of White Cells—In a 6×50 mm. tube are placed 0.5 ml. of 1.6 per cent potassium oxalate and a footed stirring rod. A finger is lanced to give a free blood flow, *e.g.* with a Bard-Parker No. 11 blade held by projection from a cork. After any residual alcohol is wiped off, the area is lightly coated with vaseline and the first blood blotted off. As rapidly as possible the paraffined pipette is filled and the blood delivered into the tube, the contents of which are at once gently but thoroughly mixed with the rod. (The pipette is rinsed with fresh oxalate but is not dried before reuse.) The tube is centrifuged within an hour at a predetermined slow speed which settles the red cells without loss of too many white cells. A safe time of centrifuging is twice the interval required just to throw the bulk of the red cells to the bottom. The centrifuge must come smoothly to a stop to avoid remixing of the sample. The turbid supernatant which contains the white cells is transferred with a Pasteur pipette to another tube of the same size. The suspension close to the red cell layer should be avoided, since this usually contains some red cells. The second tube is centrifuged (within 2 hours) at 3000 R.P.M. for 15 minutes. The clear supernatant is sucked off with the device for removing the supernatant from white blood cells, described above, and discarded. Great care is taken to remove a maximum amount of fluid without loss of the sediment. At first, a magnifying glass may prove helpful. If 90 per cent of the fluid is removed and the tube allowed to stand 5 or 10 minutes for drainage, the last 10 per cent can be removed more completely.

Ascorbic Acid Analysis—The white cells are distributed as thinly as possible by tapping the tube, and 40 c.mm. of 5 per cent trichloroacetic acid are added. For adequate extraction of ascorbic acid and acid-soluble phosphorus, it is imperative that the precipitate be well dispersed. A mechanical vibrator device (10) has been found very useful for vigorous mixing or agitation of the contents of these small tubes. After centrifuging, a 30 c.mm. aliquot of the supernatant is transferred to another 6×50 mm. tube and 10 c.mm. of the dinitrophenylhydrazine reagent are added. (The residue is saved for measuring the acid-insoluble phosphorus (see below).) After mixing and capping with parafilm or a vial stopper, the tube is incubated for 4 hours at 38° (bacteriological incubator or air bath).

After chilling in ice water, 50 c.mm. of ice-cold 65 per cent H_2SO_4 are added. Because of the viscosity the pipette should be emptied slowly. *Vigorous and thorough mixing is required* after this addition. Also, it is

advisable to tap the tubes with the finger to mix again just before reading. The light absorption is measured at 520 mμ in the spectrophotometer any time within the first 3 or 4 hours. Blanks and standards are provided by treating 30 c.mm. aliquots of 5 per cent trichloroacetic acid and of fresh 0.4 mg. per cent ascorbic acid (0.004 γ per c.mm.) in 5 per cent trichloroacetic acid in the same manner as the white cell extracts.

The ascorbic acid analysis may be deferred indefinitely if desired by storing the 30 c.mm. aliquots in well stoppered tubes at $-20°$ or colder.

Acid-Insoluble Phosphorus Analysis—After the aliquot is removed for ascorbic acid analysis, the residue is washed (within 1 hour at room temperature or 4 hours at 4°) by mixing with 0.2 to 0.3 ml. of 5 per cent trichloroacetic acid, centrifuging, and sucking off the supernatant. 30 c.mm. of 4.5 N H_2SO_4 are then added and the sample (with others in a rack) is heated for 1 or 2 hours in an oven at 95–98° to drive off excess moisture without spattering, after which ashing is accomplished by adding 10 c.mm. of 70 per cent perchloric acid and heating in an oven for 2 hours at 145–160°.

Color is developed by adding 0.2 ml. of the phosphate reagent. The samples are vigorously mixed and are mixed again by tapping with the finger just before reading.

Blanks are supplied by measuring into 6 $\times$ 50 mm. tubes 30 c.mm. of 4.5 N H_2SO_4. Standards are provided by substituting an equal volume of 0.5 mM of KH_2PO_4 in 4.5 N H_2SO_4 (0.015 micromole of P). These blanks and standards are carried through the same evaporation and ashing procedures as the white cell samples. The optical absorption is measured at 690 mμ in not less than 20 minutes after mixing. Subsequent color development is slow and occurs at a uniform rate. Some of the standards are read both before and after the unknowns to permit correction for any increase in optical density with time.

Calculation—The white cells have been found to contain an average of 3.34 mM of acid-insoluble phosphorus per 100 gm. (see the "Discussion"). Thus, the mg. of ascorbic acid per 3.34 mM of acid-insoluble phosphorus (or micrograms per 3.34 micromole) are numerically equal to the concentration per 100 gm. of white cells. Therefore

$$(1) \quad \frac{\text{Micrograms ascorbic acid in sample}}{\text{micromoles P in sample}} \times$$

$$3.34 = \text{mg. ascorbic acid per 100 gm. white cells}$$

$$(2) \quad \text{Micrograms ascorbic acid in sample} = 0.004 \times$$

$$\frac{D_{AA}}{D_{\text{standard AA}}} \times 41.5 = K_1 \times D_{AA}$$

where D_{AA} and $D_{standard\ AA}$ are the optical densities of the unknown and standard respectively, corrected for the blank. 41.5 is the sum of the 40 c.mm. acid added plus approximately 1.5 c.mm. of fluid left behind with the original white cell sediment, and 0.004 equals the micrograms of ascorbic acid per c.mm. of standard.

(3)   Micromoles P in sample = micromoles P in standard $\times$

$$0.004 \times \frac{D_P}{D_{standard\ P}} = K_2 \times D_P$$

In a series of analyses the standards and their respective optical densities are fixed, and hence Equations 1, 2, and 3 may be combined:

(4) $\dfrac{K_1 D_{AA}}{K_2 D_P} \times 3\ 34 = K_3 \dfrac{D_{AA}}{D_P}$ = mg ascorbic acid per 100 gm white cells

(The volumes of all the pipettes, except the 40 and 30 c.mm., cancel out, since they are used for both standards and unknowns.)

DISCUSSION

In the development of the above method there were two major problems: (1) the isolation of white cells from finger blood and (2) the measurement of the size of the white cell sample. Various diluting agents and anti-coagulants were investigated: heparin, citrate, heparin plus oxalate, and oxalate diluted with saline or Ringer's solution. None proved as satisfactory as isotonic oxalate for the prevention of clotting. The slightest degree of clotting will precipitate the white cells with the red blood cells. Since finger blood, even with the greatest care in collection, coagulates more rapidly than venous blood, this consideration is of first importance.

The method of isolation of the white cells is similar to that described by Gorham et al. (11), who allowed undiluted oxalated blood to stand for several hours until the red cells were partially settled and then centrifuged the supernatant. It was found expedient in adapting this principle to much smaller quantities of blood to dilute the sample with isotonic potassium oxalate. This would be expected to increase the yield of white cells and to permit more complete removal of serum without washing the cells. It also proved to be more satisfactory to remove the red cells by slow centrifugation rather than to wait for gravity to accomplish the same purpose. It is conceivable that the dilution might wash out ascorbic acid from the white cells. Actually, this does seem to occur if hypotonic oxalate is used. With isotonic oxalate, however, there appears to be no loss, as judged by the agreement recorded below, with analyses of white cells isolated on a larger scale from undiluted blood. Furthermore, the

same ascorbic acid values were found, whether the white cells were centrifuged immediately or were allowed to stand 2 or 3 hours suspended in oxalate solution.

It seemed impracticable to attempt to weigh the minute white cell samples isolated. It also proved on trial to be unsatisfactory to estimate the white cell volume from the length of the column of packed cells in a calibrated capillary tube. An attempt was, therefore, made to find a cell constituent that is relatively constant in concentration and which would not be unduly affected by the moderate contamination with red cells or fibrin which sometimes occurs. Such a substance should furnish a valid measure of the sample size. The total phosphorus or a phosphorus fraction appeared to be worthy of trial.

The total phosphorus proved to be rather constant in concentration in the white cell and as a consequence has been used as the basis for many hundreds of determinations. However, to measure the total phosphorus it was necessary to suspend the white cells evenly after isolation in order to take aliquots for both phosphate and ascorbic acid, and this was sometimes quite difficult. Both acid-soluble and acid-insoluble phosphorus were next investigated and the latter proved to be the more satisfactory. The acid-insoluble phosphorus contained in the acid-insoluble residue from the entire sample was available for analysis, whereas only part of the acid-soluble fraction could be utilized, since the rest of the acid extract would be required for the ascorbic acid determination. Furthermore, contamination of the sample with red cells would cause a greater distortion of the acid-soluble than of the acid-insoluble fraction. This reasoning is borne out by actual analyses, which proved to be more consistent when based on the acid-insoluble fraction.

Ten macro white cell samples from different individuals were isolated by centrifuging in tubes with a central narrow segment (1) and were analyzed for acid-insoluble phosphorus. An average of 3.34 mM per 100 gm. with a standard deviation of 0.25 mM per 100 gm. was found. (The individual values were 3.53, 3.16, 3.46, 2.94, 3.16, 3.36, 3.60, 3.44, 3.02, and 3.78 mM per 100 gm.)

Although the correlation between this phosphorus fraction and the wet weight is thus not perfect, it is conceivable that acid-insoluble phosphorus may be a more physiological basis of calculation than weight.

The ascorbic acid micromethod used is a modification of that previously developed for serum (12). In order to simplify the over-all procedure the conversion of ascorbic acid to dehydroascorbic acid was effected by copper instead of charcoal. The charcoal reagent is apt to cause difficulty due to floating, and its use would have necessitated several extra steps. When the charcoal is replaced, the results tend to be somewhat more uniform.

although the values observed are 2 or 3 mg. per cent higher. Apparently, charcoal removes some material (not ascorbic acid) which reacts with the reagent.

Comparative Analyses—Table I gives the data for ten white cell samples isolated from different bloods and analyzed on both a macro and micro scale for ascorbic acid. The macro samples were obtained from venous blood by the procedure described by Butler and Cushman (1). The micro samples were obtained from the finger, as described above. The ascorbic acid was determined in both cases by the dinitrophenylhydrazine procedure; the macrodeterminations were based on the weight of sample, and the microdeterminations were based on the acid-insoluble phosphorus.

The standard deviation between the two methods is 3.6 mg. per cent, as

TABLE I

Comparison of White Blood Cell Ascorbic Acid Values Measured by Macro- and Microprocedures

The values are recorded as mg. per cent.

	Serum 1	Serum 2	Serum 3	Serum 4	Serum 5	Serum 6	Serum 7	Serum 8	Serum 9	Serum 10	Average
Macro	27	30	23	25	27	24	32	26	19	25	25.8
Micro	26	27	21	31	27	31	29	25	17	26	26.0
Difference*.	−1	−3	−2	+6	0	+7	−3	−1	−2	+1	+0.2

* Standard deviation between the two methods, 3.6 mg. per cent.

judged by these data. Part of this difference is undoubtedly due to inaccuracies in the micromethod, which is based on two separate determinations, one for ascorbic acid and one for acid-insoluble phosphorus. The standard deviation of the individual microanalysis was found to be 2 mg. per cent in a series of thirty-five determinations on nine different individuals. Another source of discrepancy between the macro- and micromethods may be ascribed to differences in the acid-insoluble phosphorus content of the different white cell samples. Finally, it is difficult to isolate white cells on a macro scale and obtain entirely uniform samples. There is a possibility of different degrees of packing with variations in the accompanying fluid, and minute clots, if formed, will collect in the white cell layer and are often difficult to detect.

Reproducibility of Values for Same Individual—The white blood cells of two individuals were analyzed at intervals during the day (Table II). No significant changes were observed over a 6 hour period. Table II also

TABLE II

Hourly Measurements of Ascorbic Acid in White Blood Cells of Two Individuals

Subject A				Subject B			
Elapsed time	Isolated white blood cells (calculated)*	Ascorbic acid in white blood cells	Average	Elapsed time	Isolated white blood cells (calculated)*	Ascorbic acid in white blood cells	Average
hrs.	mg	mg per cent	mg per cent	hrs	mg.	mg. per cent	mg. per cent
0	0.23	35		0	0.34	34	
	0.24	32			0.36	33	
	0.26	30		.	0.27	33	
	0.33	33	32		0.33	27	32
0.05	0.36	32		0.05	0.24	34	
	0.30	32			0.28	33	
	0.31	31			0.24	33	
	0.24	32	32		0.32	32	33
0.10	0.38	30		0.1	0.31	33	
	0.28	31			0 31	33	
	0.26	32			0.36	33	
	0.22	29	30		0.29	33	33
0.15	0.27	32		0.15	0.26	32	
	0.39	27			0.28	32	
	0.28	33			0.28	34	33
	0.22	31	31	0.2	0.33	32	
0.20	0.23	33			0 32	32	
	0.30	30			0.32	32	32
	0.24	30		2	0.30	31	
	0.17	31	31	.	0.26	28	
2	0.35	31			0.28	29	
	0.31	31			0.13	24	
	0.33	32			0.14	24	
	0.32	29			0.14	24	27
	0.30	31		4	0.35	36	
	0.34	27	30		0.43	32	
4	0.40	26			0.31	29	
	0.46	30			0.31	26	
	0.47	30			0 31	27	
	0.35	21			0.32	33	30
	0.34	30		6	0.24	34	
	0.18	26	27		0.14	29	
6	0.29	29			0.19	29	30
	0.37	31					
	0.30	30					
	0.22	28	30				

* Calculated from acid-insoluble P, as described in the text.

indicates the mass of white cells usually isolated (0.2 to 0.3 mg.) from 0.1 to 0.15 ml. of blood.

SUMMARY

1. A method is described for the measurement of the ascorbic acid concentration in white blood cells and platelets of 0.1 ml. of blood. The cells are isolated by differential centrifugation from finger tip blood. The mass of white cells isolated are measured indirectly through a determination of the acid-insoluble phosphorus of the sample. The coefficient of variation of individual microanalyses was found to be about 8 per cent and the coefficient of variation between the micromethod described and a macromethod was about 13 per cent. Two analysts can measure 50 to 75 samples in 2 days.

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RELATION OF SCURVY TO GLUCOSE TOLERANCE TEST, LIVER GLYCOGEN, AND INSULIN CONTENT OF PANCREAS OF GUINEA PIGS

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It was reported previously that deficiency of vitamin C in guinea pigs leads to diminished glucose tolerance (1), diminished deposition of liver glycogen (2), and diminished insulin content of the pancreas (3). In those experiments, however, the guinea pigs of the control group received *ad libitum* a natural diet consisting of green grass and germinated gram (*Cicer arietinum*) and not the scorbutic diet (4) with the supplement of ascorbic acid. The experimental results obtained with scorbutic guinea pigs might, therefore, be due to the different diets consumed by the animals and not due to the specific vitamin C deficiency. Further, the results might also be due to the effect of inanition as the paired feeding technique was not employed. In the present investigation the glucose tolerance test was studied, the glycogen value of the liver was determined, and the insulin content of the pancreas was estimated in both normal and scorbutic guinea pigs. The animals received the scorbutic diet with or without the supplement of ascorbic acid and the paired feeding technique was employed.

EXPERIMENTAL

Relation of Scurvy to Glucose Tolerance Test in Guinea Pigs—Healthy male guinea pigs with an average weight of 280 gm. were fed *ad libitum* for 1 week a scorbutic diet (4) with a daily oral supplement of 5 mg. of ascorbic acid. The animals, which were growing, were divided into two groups and paired in such a way that the weights of the animals in each pair were about the same. One of the groups was fed *ad libitum* the scorbutic diet without any supplement of ascorbic acid for 21 days. The daily food consumption of each animal of this group was noted and the equivalent amount of the scorbutic diet was given to the corresponding animal of the second group, which received in addition a daily oral supplement of 5 mg. of ascorbic acid. All the animals were fed 2 drops of a concentrate of vitamins A and D twice a week during the experimental period. In the evening of the 21st day the food was removed from the cages of all the animals and the glucose tolerance test was performed the next morning as described previously (5). The results are shown in Table I.

Relation of Scurvy to Glycogen Content of Liver of Guinea Pigs—Male guinea pigs, with an average weight of 250 gm., were selected, separated into two groups, and paired as described in the previous section. They were fed the same scorbutic diet with or without the supplement of ascorbic acid for 21 days in the same way as mentioned above. In the evening of

TABLE I

Glucose Tolerance Tests in Normal and Scorbutic Guinea Pigs

Pair No.		Weight at death	Fasting blood sugar	Blood sugar after feeding glucose		
				45 min	90 min.	150 min.
		gm.	*mg per cent*	*mg per cent*	*mg per cent*	*mg. per cent*
1	Normal	400	113	222	204	177
	Scorbutic	286	124	247	300	313
2	Normal	333	106	177	177	106
	Scorbutic	250	112	298	325	313
3	Normal	436	128	187	165	156
	Scorbutic	335	131	325	247	220
4	Normal	338	138	218	192	174
	Scorbutic	263	114	284	272	174
5	Normal	320	136	213	195	134
	Scorbutic	265	174	319	292	325
6	Normal	290	97	274	247	159
	Scorbutic	224	85	325	247	235
7	Normal	325	115	177	146	130
	Scorbutic	265	130'	237	241	254
8	Normal	292	113	302	217	195
	Scorbutic	255	93	237	252	296
Average						
Normal			118 ± 3.3	221 ± 16.4	193 ± 10 6	154 ± 9 9
Scorbutic			120 ± 10.2	284 ± 13 6	272 ± 10 8	266 ± 19.4
Difference of means			2	63	79	112
Standard error of difference			10 78	21.32	15 13	21 82
t			0.2*	2 9	5.2	5 1

* Except for this value, all values of *t* are highly significant.

the 21st day the food was withdrawn from the cages. The next morning the animals were sacrificed by a blow on the head, the neck veins cut, and the livers removed, wrapped in a gauze cloth to remove adherent blood, weighed, and each liver quickly dropped in a 50 cc. centrifuge tube containing hot 30 per cent potassium hydroxide. The glycogen in the liver was precipitated by the method of Grattan and Jensen (6) and the reducing sugar in the hydrolyzed precipitate was determined by the method of

Hagedorn and Jensen (7). The glycogen values of the liver are given in Table II.

Relation of Scurvy to Insulin Content of Pancreas of Guinea Pigs—Two groups of healthy male guinea pigs, with an average weight of 250 gm.,

TABLE II

Determination of Liver Glycogen in Normal and Scorbutic Guinea Pigs

Pair No	Weight at death		Weight of liver		Glycogen per 100 gm liver	
	Normal	Scorbutic	Normal	Scorbutic	Normal	Scorbutic
	gm	gm	gm	gm.	gm.	gm.
1	304	292	6.74	9.58	1.408	0.029
2	228	186	5.64	7.80	0.833	0.056
3	233	204	7.97	5.28	1.753	0.036
4	246	217	6.32	7.75	0.794	0.183
5	335	240	5.34	7.88	1 600	0 060
6	296	258	6.38	10.62	1.809	0 011
7	238	215	5.96	4.48	3.900	0.056
8	258	242	6 68	6.50	2 453	0.108
9	260	220	5.15	9.18	2.444	0 047
10	308	286	7 14	7.52	3.178	0 008
11	288	234	7 61	5.97	2.372	0 052
12	240	209	6 47	7.55	0.695	0 109
Average			6 45	7.51	1.936 ± 0.282	0.063 ± 0.014

Difference of means		1.873
Standard error of difference .		0.282
t		6.6 (Highly significant)

TABLE III

Determination of Insulin Content of Pancreas of Guinea Pigs

	Weight of pancreas, pooled	Insulin content per gm. pancreas
	gm	I U.
Normal (10 animals)	11.91	0.45
Scorbutic (10 animals)	10.38	0.11

were selected, paired, and fed as described above for a period of 21 days. The food was removed from the cages of the animals in the evening of the 21st day and the animals were sacrificed the next morning as described in the previous section. The pancreas from the animals of each group was removed to a cooled, weighed bottle containing acid alcohol of the following

composition: alcohol 75 parts, distilled water 25 parts, and concentrated
hydrochloric acid 1.5 parts. The second weighing of the bottle gave the
weight of the pancreatic tissue. The insulin in the pooled pancreas of
each group of animals was extracted by the method of Best, Haist, and
Ridout (8). The potency of insulin in these extracts was quantitatively
estimated by the rabbit assay method of Marks (9). The results are given
in Table III.

DISCUSSION

Unlike those reported by Sigal and King (10) and Murray and Morgan
(11), the fasting blood sugar values of normal and scorbutic guinea pigs did
not differ significantly. Blood sugar values of scorbutic guinea pigs 45,
90, and 150 minutes after the feeding of glucose were significantly higher
than the corresponding values of the paired fed control animals. The
glucose tolerance was, therefore, definitely lowered in all the scorbutic
guinea pigs. The glycogen content of the liver was markedly lowered in
all the scorbutic guinea pigs and the results were statistically significant
(Table II). The insulin content of the pancreas of scorbutic guinea pigs
was markedly decreased when compared with that of the paired fed normal
control animals (Table III). In our previous experiments (3) in which
the normal animals received the diet *ad libitum*, the insulin content was 0.6
I. U. per gm. of pancreas. This value was a little higher compared to our
present findings. Inanition, therefore, slightly lowers the insulin content
of the pancreas. This was also reported by Best *et al.* (8) in experiments
with rats. The decrease in the insulin content of the pancreas in scurvy is,
therefore, mainly due to the effect of vitamin C deficiency. In our pre-
vious experiments (3) the animals of the scorbutic group received the
scorbutic diet for a period of 24 to 28 days. The insulin content of the
pancreas of those animals was 0.08 unit per gm. of pancreas. In the
present investigation the animals of the scorbutic group received the scor-
butic diet for 21 days only and the insulin content was 0.11 unit per gm. of
pancreas, a little higher than our previous result. These findings indicate
that the insulin content of pancreas gradually diminishes with the progress
of scurvy. Though the adrenalin content of the adrenal glands of guinea
pigs markedly increased when the animals developed scurvy (4, 5), scorbutic
guinea pigs with demedullated adrenals showed lowered glucose tolerance,
as did the scorbutic guinea pigs with intact adrenals (5). The lowered
glucose tolerance and the lowered glycogen content of the liver as observed
in scorbutic guinea pigs, therefore, might be explained by the diminished
insulin content of the pancreas. Vitamin C seems to stimulate the islands
of Langerhans for the normal secretion of insulin. The deficiency of this
vitamin was found to cause degranulations in the β-cells of the pancreas

of guinea pigs (12). Like the guinea pig, the human cannot synthesize vitamin C and a chronic deficiency of vitamin C might cause degenerative changes in the islands of Langerhans, resulting in diabetes mellitus.

SUMMARY

1. The effect of vitamin C deficiency on the glucose tolerance, on the glycogen content of the liver, and on the insulin content of the pancreas was studied in guinea pigs by a paired feeding technique.

2. Glucose tolerance was significantly lowered in scorbutic guinea pigs.

3. The glycogen value of the liver was significantly diminished in scorbutic animals.

4. The insulin content of the pancreas of scorbutic guinea pigs was diminished to about one-fourth of the normal value.

5. The insulin content of the pancreas was found to diminish with the progress of scurvy, and inanition was also found to lower slightly the insulin content of the pancreas.

6. It has been suggested that the disturbed carbohydrate metabolism as seen in scurvy is due to a deficiency of insulin secretion and a chronic deficiency of this vitamin may be one of the etiological factors of diabetes mellitus in human subjects.

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THE METABOLISM OF PHENOXYACETIC ACID, ITS HOMO-LOGUES, AND SOME MONOCHLOROPHENOXYACETIC ACIDS. NEW EXAMPLES OF β OXIDATION

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The biological behavior of benzoic acid and its homologue, phenylacetic acid, has been the subject of many investigations. Phenylacetic acid, like benzoic acid, conjugates with glycine in the organism of the common laboratory animals (dog, cat, rabbit) to form phenaceturic acid, the homologue of hippuric acid (1). Phenoxyacetic acid is a compound which differs from phenylacetic acid in that there is an ether linkage between the benzene ring and the aliphatic side chain (C_6H_5—O—CH_2—COOH). It was of interest to determine the biological behavior of phenoxyacetic acid and some of its higher homologues.

When glycine is fed to the rabbit with phenylacetic acid, the rate of excretion as the conjugation product, phenaceturic acid, is increased. If phenoxyacetic acid were conjugated with glycine, as is phenylacetic acid, the administration of glycine with the acid should increase both the extent of conjugation and the rate of excretion of the conjugate. No evidence of conjugation with glycine was obtained either when the acid was fed alone or with glycine. No evidence of conjugation with glucuronic acid could be obtained. The o- and p-monochlorophenoxyacetic acids were also studied. The halogen derivatives are of interest in view of the use of the dichloro derivative of phenoxyacetic acid as a weed killer in agriculture. The activity of phenoxyacetic acid and its monochloro derivatives as plant hormones has been studied by Zimmerman (2).

In his studies on biological oxidation of fatty acids (3), Knoop employed fatty acids in which a phenyl group was substituted in the carbon chain at the ω- or terminal carbon atom, and from these observations developed the well known theory of β oxidation. Carter (4) has also used similarly substituted fatty acids to study the mechanism of fatty acid oxidation. The failure of oxidation of phenoxyacetic acid might make this compound of value in the study of the oxidation of its higher homologues, γ-phenoxybutyric and ε-phenoxycaproic acids, since β oxidation of these acids should result in the formation of phenoxyacetic acid which escapes further oxidation. These homologues of phenoxyacetic acid were synthesized

and fed to rabbits. The unchanged acids could not be found in the urine, but phenoxyacetic acid could be isolated from the urine by the formation of the S-benzylthiuronium salt. Since phenoxyacetic acid could originate only from the oxidation of the acids fed, the experiments afford new evidence of the β oxidation of fatty acids.

<div align="center">EXPERIMENTAL</div>

Compounds Used—Phenoxyacetic acid and the *o*- and *p*-monochlorophenoxyacetic acids were obtained through the courtesy of Mr. W. M. Alles of the Dow Chemical Company, to whom we wish to express our appreciation of his cooperation. The purity of these acids was established by the determination of their melting point and of their neutral equivalents. γ-Phenoxybutyric acid was synthesized by the method of Bentley, Haworth, and Perkins (5), and ε-phenoxycaproic acid by the method of von Braun (6). The ε-phenoxycaproic acid, even after three recrystallizations from petroleum ether, melted at 55–56° (uncorrected), a melting point definitely lower than that of 71°, as reported by von Braun.

Since it was anticipated that if phenoxyacetic acid were conjugated with glycine, the conjugated acid, phenoxyacetylglycine,[1] would be excreted, it was necessary to prepare this acid whose synthesis has not been carried out previously, so far as is known to us. Phenoxyacetyl chloride was prepared by the action of thionyl chloride on phenoxyacetic acid (7). The acid chloride was then coupled with glycine by the usual Schotten-Baumann procedure. No difficulties were encountered in the synthesis. After repeated recrystallization from hot water and drying over phosphorus pentoxide, the acid melted at 115–116° (uncorrected), had a neutral equivalent of 204.6 and 208.2 (theoretical 209), and a nitrogen content of 6.63 per cent (theoretical 6.69 per cent).

In previous studies from this laboratory (8), we have found the S-benzylthiuronium salts of value in the identification of derivatives of fatty acids. We have prepared these salts of the acids with which we are concerned in the present investigation, applying the conditions for their preparation which are outlined by Donleavy (9). Since, to our knowledge, the preparation and properties of these particular salts have not been described, we are presenting the melting points and nitrogen contents of the derivatives of the pure acids, as well as of those isolated from the experimental urines in Table I. For the isolation and characterization of phenoxyacetic acid and related compounds from urine, the acidified urine has been extracted by ether in a continuous extractor, and the ether has been removed from the extract by evaporation.

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[1] The name, "phenoxyaceturic acid," will be used for convenience throughout this discussion in place of phenoxyacetylglycine. This nomenclature is similar to that of phenaceturic acid for phenylacetylglycine.

The residual material was neutralized by sodium hydroxide, the sodium salts were dissolved in hot alcohol, and the salts were precipitated as described (8, 9). The derivatives prepared from the ether extracts of urine were usually of satisfactory purity after one recrystallization (8).

TABLE I

Analyses and Melting Points of S-Benzylthiuronium Salts of Phenoxyacetic Acid, o- and p-Monochlorophenoxyacetic Acids Prepared from Pure Acids and Isolated from Experimental Urines

In all the experiments mixed melting points of the salts isolated from the experimental urines and of the salts of the pure acid were determined also. No depression of the melting points were observed. All melting points are uncorrected.

Acid fed				M.p.	Nitrogen	
		Isolated as acid fed				
	(a)	(b)	$\frac{b}{a} \times 100$		Found	Calculated
	mg.	*mg.*		*°C.*	*per cent*	*per cent*
A. Pure compounds						
Phenoxyacetic				170	8.82	8.83
o-Chlorophenoxyacetic				159 –159.5	7.99	8.09
p-Chlorophenoxyacetic				183.5–184	7.99	8.09
γ-Phenoxybutyric				139 –140		
B. Isolated from urine						
Phenoxyacetic	560	286	51	170		
"	700	340	48	170		
"	600	257	43	170 –171	8.73	8.83
o-Chlorophenoxyacetic	400	216	54	158	8.06	8.09
"	300	133	44	158 –159		
p-Chlorophenoxyacetic	486	193	41	183 –184		
"	300	106	35	183 –184	7.94	8.09
γ-Phenoxybutyric	400	44*	13†	170	8.72	8.83
"	350	40	14†	169		
"	500	160	38†	170	8.73	8.83
ε-Phenoxycaproic	420	145*	23†	169	8.62	8.83
"	420	125	20†	170	8.96	8.83

* Carbon and hydrogen determinations made on these salts showed 60.39 and 60.55 per cent of carbon and 5.77 and 5.93 per cent of hydrogen on the respective samples indicated. Theoretical for the salt of phenoxyacetic acid, 60.31 and 5.66 per cent of carbon and hydrogen respectively.

† The salt obtained was the salt of phenoxyacetic acid, as shown by the melting points and analyses. The percentage is calculated as the percentage of the phenoxyacetic acid which could be derived by oxidation of the amount of acid fed.

Methods of Analysis—Since phenoxyaceturic acid was a possible urinary component after the administration of phenoxyacetic acid, it was necessary to study methods for its determination. In the Griffith method for the determination of the similar compound, hippuric acid, the acidified urine

is extracted with ether and the nitrogen content of the extract is determined as a measure of the hippuric acid content after the destruction of any extracted urea by the use of hypobromite (10). We have found that phenoxyacetic acid added to normal rabbit urine may be satisfactorily recovered (97.6, 98.1, and 99.0 per cent in three consecutive tests) in this way. It was also desirable to determine total phenoxyacetic acid in urine. The procedure used by Kingsbury and Swanson (11) for the estimation of total benzoic acid, when applied to phenoxyacetic acid, resulted in the formation of some substance (presumably a nitrophenol derivative) which was not completely extracted by the chloroform and, in addition, was only partially reextracted from the chloroform by the salt solution. The yellow color of this material interfered with the titration of the chloroform extract with alkali.

A colorimetric procedure was developed which was based on the oxidative cleavage of phenoxyacetic acid by nitric acid to yield a dinitrophenol. The intensity of the color of the sodium salt of dinitrophenol was measured with the aid of the Evelyn photoelectric colorimeter.

The 6 or 18 hour specimens of rabbit urine were diluted to 100 and 200 ml. respectively, and 5 ml. of this diluted urine were further diluted to 500 ml. 2 ml. of the urine as finally diluted were placed in a micro-Kjeldahl flask calibrated at 12 ml. A glass bead was added and the contents of the flask were reduced nearly to dryness by heating, preferably on a Kjeldahl micro digestion rack. Heating was stopped just before the last drop of liquid disappeared from beneath the bead. 3 ml. of concentrated nitric acid were added and the acid was evaporated by heating on the rack until a small amount of acid remained in the flask. It was necessary to exercise caution at this point, since, if the residue was allowed to become entirely dry, the contents sometimes exploded and low results were obtained. After cooling, 5 ml. of distilled water were added, followed by 3 ml. of 25 per cent sodium hydroxide. After dilution to the mark with distilled water and careful mixing, the intensity of the color which developed immediately was measured in an Evelyn photoelectric colorimeter (Filter 420). The readings were converted to mg. with the aid of a calibration curve, obtained by the use of the above procedure with 2 ml. of normal diluted rabbit urine to which known amounts of phenoxyacetic acid had been added. Aqueous solutions of the acid could not be used in the establishment of the calibration curves, since there was present in rabbit urine some substance, in the presence of which the color of the sodium salt of the dinitrophenol was intensified. By the use of this procedure, it was possible to recover from 93 to 99 per cent of added phenoxyacetic acid. Similar calibration curves were prepared for the monochloro derivatives, for phenoxyaceturic acid (which gave, as expected,

essentially the same values as phenoxyacetic acid), and for γ-phenoxy-butyric acid. With the monochloro compounds, it was necessary to use fuming nitric acid to split the ether linkage.

Glucuronic acid was determined colorimetrically by the procedure already described from this laboratory (8) with the use of the photoelectric colorimeter. Creatinine was determined as a check on the completeness of the collection of the sample, but the results are not recorded.

Experimental Procedure—Male rabbits of 2 to 3 kilos of body weight, maintained on constant diets of oats and cabbage, served as experimental animals. Urine was collected by gentle pressure on the bladder and was separated into 6 and 18 hour fractions. The sodium salts of the acids in aqueous solution were fed by stomach tubes. Phenoxyacetic acid was first fed at a level of 200 mg. per kilo, but later a somewhat smaller dosage of 100 mg. per kilo was fed. When glycine was also administered, it was given in amounts corresponding to three equivalents of the acid fed.

Since, so far as is known to us, reports of the toxicity of the monochloro derivatives of phenoxyacetic acid are not available, preliminary experiments with these acids were carried out. Male white rats, 200 gm. in weight, received approximately 200, 400, and 800 mg. per kilo orally for 3 consecutive days with no signs of toxicity. The animals consumed their daily food rations completely. Rabbits which received *p*-mono-phenoxychloroacetic acid at the 200 mg. per kilo level showed a marked hematuria and proteinuria which persisted for approximately 72 hours after the feeding. In subsequent feeding experiments with rabbits, the monochlorophenoxyacetic acids were fed at a level of 125 mg. per kilo (equivalent to 100 mg. of phenoxyacetic acid).

DISCUSSION

The results of the experiments in which phenoxyacetic acid was fed may be summarized as follows. There was no evidence that phenoxyacetic acid was conjugated to a significant degree with either glucuronic acid or with glycine (even when extra glycine in considerable amounts was supplied in the diet). These results, as far as conjugation with glycine to give phenoxyaceturic acid is concerned, are in harmony with the limited earlier and essentially qualitative experiments with man (12, 13) and the dog (13). The excretion of "total" phenoxyacetic acid by the kidneys was rapid, 60 per cent of the amount ingested being excreted within 6 hours (range, 44 to 72 per cent in nine experiments), and 96 per cent in 24 hours (range, 82 to 105 per cent). It is of interest to compare the rate of excretion of phenoxyacetic acid with that of phenylacetic acid in the same species, even though the dose per kilo was significantly greater in the experiments with the latter (1). The excretion of phenylacetic acid, both total

and combined as phenaceturic acid, was slow and the excretion continued over a period of several days.

It was also possible to isolate phenoxyacetic acid from the experimental urines as the S-benzylthiuronium salts[2] by the procedure already discussed. As shown in Table I, from 43 to 51 per cent of the acid fed could be isolated thus as a derivative of satisfactory purity, as shown by melting points and analyses for nitrogen.

When o-monochlorophenoxyacetic acid was fed either with or without glycine, there was no evidence of conjugation, and prompt excretion by the kidneys was observed, i.e. in four experiments from 50 to 72 per cent of the acid administered in 6 hours. These results are similar to those obtained with the unsubstituted phenoxyacetic acid and are in sharp contrast to the results obtained after the oral administration of the isomeric p-monochlorophenoxyacetic acid. Here also no conjugation was observed, but the excretion in the first 6 hours was so low (13 to 15 per cent of the administered acid in four experiments) that the values approached the lower limits of accuracy of the analytical procedures used. With the dosage used (125 mg. per kilo) there were no signs of toxicity, although the possibility of delayed absorption from the intestine or renal injury as factors in the lower rate of excretion cannot be ruled out. In the 24 hour period the total excretion was essentially the same for both the mono-chloro acids, an excretion corresponding to 70 to 90 per cent of the amount administered. It was possible to isolate each of these acids from the experimental urines as the SBT salts. The data are presented in Table I.

Since it was shown that phenoxyacetic acid was excreted unchanged, it was of interest to determine whether the higher homologues of phenoxyacetic acid would be excreted unchanged or whether, by oxidation of the side chain, they would be converted to lower homologues or to phenoxyacetic acid itself. When γ-phenoxybutyric acid was fed with or without glycine, the rate of excretion of metabolic products was similar to that observed after phenoxyacetic acid (53 and 58 per cent in 6 hours in two experiments), with no evidence of conjugation with either glycine or glucuronic acid. That oxidation to yield phenoxyacetic acid had occurred was shown by the isolation of the SBT salt of phenoxyacetic acid from the experimental urines. As shown in Table I, the amounts of the salt isolated in three experiments were equivalent to 13, 14, and 38 per cent of the theoretical amount of phenoxyacetic acid which could be obtained by β oxidation of the γ-phenoxybutyric acid fed.

After ε-phenoxycaproic acid had been fed to two rabbits, 20 and 23 per cent of the theoretical amount of the SBT salt of phenoxyacetic acid was obtained from the urine. Melting points, mixed melting points, and

[2] For convenience, these salts will be referred to hereafter as SBT salts.

analyses for nitrogen were satisfactory (Table I). However, as added proof, one sample of the phenoxyacetic acid salt isolated after the administration of γ-phenoxybutyric acid and one sample obtained after ε-phenoxycaproic acid were analyzed for carbon and hydrogen, as shown in the foot-notes to Table I. All the evidence indicates clearly that the oxidation, presumably β oxidation, of the acids has occurred, and that the product of oxidation, phenoxyacetic acid, is excreted in the urine. Since the preparation of the SBT salts of other acids from urine has been estimated to yield from 50 to 60 per cent of the theoretical amount (8), it is believed that the amount of phenoxyacetic acid actually present in the urine represents a significant proportion of the acid fed.

Although no conjugation of phenoxyacetic acid with glycine was observed, it was of interest to study the fate of the potential conjugation product, phenoxyaceturic acid, which we had synthesized as already described. The acid was administered orally or subcutaneously to rabbits in amounts equivalent to 100 mg. of phenoxyacetic acid per kilo and the total phenoxyacetic acid and the phenoxyacetic acid as phenoxyaceturic acid were determined in the 6 and 18 hour urines. The difference between total and conjugated phenoxyacetic acid was an index of the amount of phenoxyaceturic acid hydrolyzed. With two exceptions (one oral and one subcutaneous administration), significant hydrolysis was observed, a hydrolysis which was greater in the 18 hour samples than in the 6 hour. That this hydrolysis was not due to the activity of the intestinal microflora was shown by the observation that in four experiments with subcutaneous injection hydrolysis was observed in three of the four urines collected after 6 hours and in all four of the 18 hour specimens. From the urine of one animal which had received 500 mg. of phenoxyaceturic acid orally, the equivalent of 108 mg. of phenoxyacetic acid was isolated as the SBT salt. This corresponded to about 30 per cent of the phenoxyacetic acid which could be obtained by complete hydrolysis of the phenoxyaceturic acid fed. The melting point, mixed melting point, and nitrogen content (8.75 per cent) corresponded to those of known samples of the SBT salt of phenoxyacetic acid.

The hydrolytic cleavage of phenaceturic acid observed was not due to changes in the urine after voiding. When urine samples containing phenaceturic acid were incubated at room temperature, hydrolysis was slight, less than 6 per cent in 24 hours. A similar study of hippuric acid in urine showed a hydrolysis of more than 40 per cent in two experiments.

Histozyme, a non-specific enzyme, which effects the hydrolysis of hippuric acid, is known to occur in certain animal tissues, notably the kidney. So (14) has observed that phenaceturic acid was readily hydrolyzed by a purified histozyme preparation from pig liver, less readily by a similar

preparation from pig kidney.   We have prepared partially purified his-
tozyme (acetone-dried preparations) from rabbit kidney by the method
of So (14) and have studied its action on hippuric and phenoxyaceturic
acid in parallel experiments.

Hippuric or phenoxyaceturic acid was determined by the use of the
Griffith method (10) before and after incubation.   The decrease after
incubation represented the amount hydrolyzed.   The details are presented

TABLE II

Hydrolysis of Hippuric and Phenoxyaceturic Acids by Histozyme of Rabbit Kidney

Test solutions included 25 ml of phosphate buffer (pH 6 8), 200 mg of enzyme
powder,* and the amount of substrate indicated   The mixed materials were diluted
to 50 ml with distilled water   A small crystal of thymol was added, and the solu-
tion was incubated for 24 hours at 36–37°.   Blank values have been subtracted

Experiment No	Time	Substrate		Hydrolysis
	hrs		*mg*	*per cent*
1	0	Hippuric acid	108.0	
	24	" "	88 2	18 3
2	0	Phenoxyaceturic acid	101 5	
	24	" "	32.6	68.9
3	0	Hippuric acid	126.4	
	24	" "	121.3	3.25
4	0	Phenoxyaceturic acid	100.8	
	24	" "	66.7	33.8
5	0	Hippuric acid	143 4	
	24	" "	142 2	0 9
6	0	Phenoxyaceturic acid	147 4	
	24	" "	100 3	32.1
7	0	Hippuric acid	131 1	
	24	" "	122 8	7 1
8	0	Phenoxyaceturic acid	75 3	
	24	" "	47 3	37.2

* Two histozyme preparations were used   Preparation 1 in Experiments 1 and
2, and Preparation 2 in the other experiments

in Table II.   Preparation 1 of the kidney powder was more effective in
the hydrolysis of both hippuric and phenoxyaceturic acid than was Prepa-
ration 2, which hydrolyzed hippuric acid very slightly.   However, in
each of the four sets of experiments, the extent of hydrolysis of phenoxy-
aceturic acid was clearly greater than was that of hippuric acid.   The
data suggest that the hydrolysis of phenoxyaceturic acid, observed after
both oral and parenteral administration, may be related to the activity
of the histozyme of the kidney.   It is, of course, possible that similar
enzymatic activity may be associated with other organs.

SUMMARY

The S-benzylthiuronium salts of phenoxyacetic acid and some of its derivatives and homologues have been prepared and their properties described. These salts have been useful for the identification of the phenoxy acids and their products of metabolism in urine. Phenoxy-acetylglycine ("phenoxyaceturic" acid) has been synthesized. When this compound was fed or injected into rabbits, some hydrolysis occurred, as evidenced by the excretion of phenoxyacetic acid in the urine. The presence of an enzyme which hydrolyzed phenoxyaceturic acid more readily than hippuric acid was demonstrated in "histozyme" preparations from rabbit kidneys (acetone-dried tissue).

When phenoxyacetic and o- and p-monochlorophenoxyacetic acids were fed to rabbits, they were excreted in the urine. No evidence of conjugation with either glycine (phenoxyaceturic acids) or glucuronic acid was obtained.

When γ-phenoxybutyric acid and ϵ-phenoxycaproic acid were fed, only phenoxyacetic acid was isolated from the urine. This is further evidence in support of the Knoop theory of β oxidation of fatty acids, since the phenoxyacetic acid is believed to be derived from the higher homologues by β oxidation.

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STUDIES ON CHOLINESTERASE*

III. PURIFICATION OF THE ENZYME FROM ELECTRIC TISSUE BY FRACTIONAL AMMONIUM SULFATE PRECIPITATION

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It has been shown that nerve and muscle tissues contain an esterase easily distinguished from all other esterases by its high relative specificity for acetylcholine (10). This enzyme, cholinesterase, is present in all types of nerves and muscles throughout the animal kingdom, and has been found in the heads of·Tubularia, a hydrozoan coelenterate, the lowest animal form to possess neuromuscular tissue comparable to that of higher animals (5). Substances which are inhibitors of the enzyme also abolish conduction in nerve and muscle (2). This abolition is reversible when the enzyme inhibition is also reversible, whereas irreversible inhibition of the enzyme is paralleled by irreversible inhibition of conduction. A striking parallelism has been obtained between the behavior of the chemical and electrical processes if their irreversibility is tested as a function of time and temperature (3, 4, 6). In connection with a great number of other facts, the evidence appears to be conclusive that the activity of cholinesterase is inseparably associated with the conduction of impulses along nerve and muscle fibers (7, 8).

It is obvious that an enzyme of such apparent importance in the functioning of nerve and muscle is of great interest and that more information is desirable concerning its chemical and physicochemical properties. It is useful to obtain the enzyme in purified form in order to facilitate detailed studies concerning its chemical and physicochemical properties. In this paper will be described a procedure by which cholinesterase of a high degree of purity has been obtained by fractional ammonium sulfate precipitation of extracts from the electric organ of *Electrophorus electricus*. The same procedure always yields nearly identical results. Studies of the physicochemical properties of the enzyme will be reported in subsequent papers.

Electric tissue has been selected because it splits per hour amounts of acetylcholine equivalent to 1 to 3 times its own weight. This extraordinarily high concentration of the enzyme is particularly interesting in view of the low protein (2 per cent) and high water content (92 per cent). It is rare to find a material which offers itself so favorably as a source for

enzyme purification.   It was found, in 1938, that cholinesterase may be easily extracted from these organs and obtained in cell-free solution by homogenization and centrifugation (9).   Certain properties of this enzyme have been described.   It has also been shown that the esterase in these organs is exclusively specific cholinesterase (10).

Methods

The manometric method was used for the determination of the enzyme activity in the usual way (10).   The buffer solution in the vessel contained 0.13 M NaCl, 0.04 M $MgCl_2$, and 0.025 M $NaHCO_3$ in final concentration. The substrate was placed in the side bulb in 0.1 cc., its final concentration after mixing being 0.018 M.   If the enzyme solution was diluted to a very high degree, gelatin was added (0.1 per cent final concentration), since it was found that the enzyme in high dilution becomes unstable.

The protein content was determined by drying and weighing.   1 cc. of the enzyme solution was put into a small centrifuge tube of 3 cc. capacity and of known weight (between 4.0 and 4.5 gm.).   1 cc. of 20 per cent trichloroacetic acid was added.   The precipitate was left to settle overnight. The tube was centrifuged at 4000 R.P.M. for about 10 minutes and the supernatant was discarded.   The precipitate was washed twice with 2 cc. of distilled water.   After the supernatant of the second washing had been removed, the tube was kept in a drying oven at 110° and weighed on the following day.

The dialysis of the purified cholinesterase solutions was carried out in the following manner: The enzyme, after solution in secondary phosphate plus phosphate buffer, was poured into a bag made of cellophane casing, a small glass bead was added, and the open end then tied.   Allowance was made for a sufficiently large air bubble to cause stirring of the solution. The cellophane bag was then attached to an eccentric stirrer and immersed in a 3 liter jar.   The stirrer was rotated by means of a small gear reduction motor.

For the 1st day dialysis was carried out against 0.1 M Na_2HPO_4.   The outer dialysis fluid was changed every $1\frac{1}{2}$ hours.   In all six to seven changes of secondary phosphate (3 liters each) were used.   The outer dialysis fluid was changed to 0.1 M NaCl, 0.015 M $MgCl_2$, 0.015 M phosphate buffer, pH 7.4, in the final change.

During the 2nd and 3rd days, the dialysis was continued against the latter solution, changes being made every 2 hours.   In all, six to seven changes were made each day.

Results

Preparation of Material—The electric organ contains an appreciable amount of mucin (1), which proved to be most disturbing in earlier attempts

at purification of cholinesterase extracted from this tissue. It could, however, be easily removed in the following manner: the tissue was cut into small pieces and allowed to remain in the refrigerator under toluene for 1 month. Each day there appears some exudate which has a high mucin content. The exudate is discarded periodically and finally, at the end of 4 to 5 weeks, the tissue contains very little mucin.

In the preparation to be described here, 10.05 kilos of electric tissue from nine electric eels (*Electrophorus electricus*) were treated in the way mentioned above. At the end of 1 month, 6.44 liters of exudate had been removed. The remaining 3.6 kilos of tissue were ground in a Waring blendor with 9.2 liters of 5 per cent ammonium sulfate. During the first grinding, 250 cc. of ammonium sulfate were added to each 100 gm. portion

TABLE I.

Cholinesterase Activities and Protein Contents of Solutions Obtained during Purification of Enzyme from Electric Tissue of Electrophorus electricus

The last column indicates the enzyme activity per unit of protein (A_P = mg. of acetylcholine split per hour per mg. of protein). Preparation 8 has been obtained by high speed centrifugation of a solution similar to Preparation 7.

Preparation No	Dilution used	CO₂ output	ACh split	Protein	A_P
		c mm. per hr.	*gm. per cc. per hr*	*mg. per cc.*	
1	1:300	392	0.950	3.3	288
2	1:1,200	360	3.5	8.0	438
3	1:5,000	301	12.2	4.3	2,810
4	1:10,000	220	17.8	4 9	3,630
5	1:10,000	368	29.8	6.1	4,890
6	1:10,000	442	35.8	4 8	7,460
7	1:20,000	303	49.2	2.3	21,400
8	1:200,000	323	524 0	7.0	75,000

of tissue. The second extraction was carried out with two-thirds the volume of the first extraction. In this way, 10.24 liters of solution were obtained. The enzyme activity and the protein content of this solution and of those obtained during the course of purification are recorded in Table I. 1 cc. of this solution was able to split 0.95 gm. of acetylcholine per hour (Table I, Preparation 1). The total amount of solution prepared was then able to split 9.7 kilos of acetylcholine per hour. 1 mg. of protein of this solution could split 288 mg. of acetylcholine per hour. The activity of the preparation per unit of protein will be called A_P. In this instance A_P was consequently 288.

Purification of Cholinesterase Solution

First Step of Purification—Solid ammonium sulfate was added to the 10.24 liters of solution in sufficient quantity to make the concentration of

ammonium sulfate 15 per cent. The precipitate formed was centrifuged and discarded, since it was known that at a concentration of 15 per cent ammonium sulfate only negligible amounts of cholinesterase are precipitated. To the supernatant solution was added solid ammonium sulfate to increase the concentration to 40 per cent. The precipitate was filtered by suction. The filter paper was comminuted and suspended in 1350 cc. of 5 per cent ammonium sulfate. The pH was adjusted to 7.1. The paper was filtered off through gauze and washed first in 750 cc. of 5 per cent ammonium sulfate (pH 7.0) and then a second time in 400 cc. of 5 per cent ammonium sulfate. Each of these washings was then combined with the original solution.

2.54 liters of solution were obtained in this way. 1 cc. was able to split 3.5 gm. of acetylcholine per hour. The activity of the whole amount was 8.9 kilos. The A_P was 438 (Table I, Preparation 2). The solution was centrifuged at 2000 R.P.M. for 13 minutes to remove the mucin-like substance floating in the solution. The enzyme activity was unaffected by this operation. About one-half of this solution, 1.25 liters, was precipitated by the addition of sufficient saturated ammonium sulfate to bring the concentration up to 19 per cent. At this concentration of ammonium sulfate some loss of cholinesterase occurs, but, at the same time, much of the protein is removed and a considerable degree of purification is obtained. The pH was adjusted to 5.82. The solution was left in the refrigerator overnight in order to allow the precipitate to digest and settle. The precipitate was centrifuged at 2000 R.P.M. in an anglehead centrifuge and washed first with 450 cc and then with 300 cc. of 19 per cent ammonium sulfate (pH 5.82). The washings were combined. The supernatant fluid and washings were precipitated separately at 29 per cent ammonium sulfate by the addition of saturated ammonium sulfate (pH 5.62). Most of the enzyme is precipitated at this concentration of ammonium sulfate at the slightly acid pH used.

The solution was then centrifuged at 2000 R.P.M. The supernatant was discarded. Three-fourths of the protein was dissolved in 113 cc. of 5 per cent ammonium sulfate (pH was adjusted to 7.3). The remaining fourth of the total protein was dissolved in 25 cc. of 0.1 M NaCl, 0.015 M $MgCl_2$, 0.015 M phosphate buffer, pH 7.4, plus 5 cc. of 0.1 M Na_2HPO_4. This solution was dialyzed for 3 days. At the end of the dialysis 52 cc. of solution were obtained. 1 cc. of this solution was able to split 12.2 gm. of acetylcholine per hour. The activity of the whole amount was 634 gm. of acetylcholine per hour. The A_P was 2810 (Table I, Preparation 3).

The remaining 1160 cc. of solution with an A_P of 438 were precipitated

successively at 19 and 29 per cent ammonium sulfate and treated in a manner similar to the previous fraction. The precipitate obtained at 29 per cent was dissolved in 120 cc. of 5 per cent ammonium sulfate and combined with the corresponding solution of the first fraction in which three-fourths of the protein had been dissolved in 113 cc. of 5 per cent ammonium sulfate. The volume of the two solutions combined was 300 cc., 1 cc. of which was capable of splitting 17.8 gm. of acetylcholine per hour. The activity of the whole volume was 5.3 kilos. A_P was 3630 (Table I, Preparation 4).

Second Step of Purification—This solution was precipitated with 19 per cent ammonium sulfate (pH 5.83). The precipitate was allowed to settle for 2 hours, was centrifuged at 2000 r.p.m., and the precipitate discarded. To the supernatant was added saturated ammonium sulfate to 28 per cent (pH 5.57). The precipitate was centrifuged on the next day and dissolved in 100 cc. of 5 per cent ammonium sulfate (pH 7.0). 148 cc. of a solution were obtained and were able to split 29.8 gm. of acetylcholine per hour per cc. The activity of the whole volume was 4.41 kilos. A_P was 4890 (Table I, Preparation 5).

40 cc. of this solution were precipitated at 28 per cent with saturated ammonium sulfate (pH 5.66). The precipitate was allowed to remain in the refrigerator overnight to settle and then centrifuged at 5000 r.p.m. for 15 minutes. The precipitate was dissolved in 6 cc. of 0.1 M NaCl, 0.015 M $MgCl_2$, 0.015 M phosphate buffer, pH 7.4, plus 6 cc. of 0.1 M Na_2HPO_4. 17 cc. of this solution were dialyzed for 3 days. 19 cc. of a solution were obtained. 1 cc. of this solution was able to split 35.8 gm. of acetylcholine per hour. The activity of the whole volume was consequently 680 gm. A_P was 7460 (Table I, Preparation 6).

Third Step of Purification—105 cc. of solution with A_P 4890 were precipitated at 20.5 per cent ammonium sulfate and centrifuged at 5000 r.p.m. after 3 hours. The supernatant was saved and the precipitate was washed three times with 30 cc. of 20.5 per cent ammonium sulfate (pH 6.2). The supernatant and washings (kept separated from the supernatant) were precipitated at 27 per cent with saturated ammonium sulfate. The precipitate was centrifuged at 3500 r.p.m. in the refrigerated centrifuge at 10°. The combined precipitates were dissolved in 35 cc. of 1 per cent ammonium sulfate.

The above solution was then precipitated at 27 per cent with saturated ammonium sulfate (pH 5.61) and allowed to remain in the refrigerator for 3 hours. This was centrifuged in the cold centrifuge as above, and the precipitate dissolved in 7.5 cc. of 0.1 M NaCl, 0.015 M $MgCl_2$, 0.015 M phosphate buffer, pH 7.4, plus 7.5 cc. of 0.1 M Na_2HPO_4. The solution

was dialyzed for 3 days. This and the other dialyzed solutions were used for ultracentrifuge studies, which will be described elsewhere.

28.5 cc. of solution were obtained. 1 cc. was able to split 49.2 gm. of acetylcholine per hour. The activity of the whole amount was 1.40 kilos. A_P was 21,400 (Table I, Preparation 7).

Specificity Pattern of Purified Cholinesterase—If the rates of hydrolysis of different esters by a solution of cholinesterase purified from electric tissue are tested, it is found that the same pattern is obtained as with the freshly prepared suspension (10). The highest A_P at which the pattern was previously tested was 3000. It appeared of interest to see whether or not a still higher degree of purification affects the pattern observed earlier. In Table II the data obtained with preparations of an increasing degree of

TABLE II

Specificity of Cholinesterase from Electric Tissue of Electrophorus electricus, Tested by Rate of Hydrolysis of Different Esters, in Course of Purification

qx = the hydrolysis rate of Substrate x in per cent of that of acetylcholine.
Pr = propionylcholine, Bu = butyrylcholine, Me = acetyl-β-methylcholine (mecholyl). q of benzoylcholine, tributyrine, and methylbutyrate was always 0.

A_P	ACh split	qx		
		qPr	qBu	qMe
	c mm per hr.			
270	335	97	1	22
1,365	311	101	3	31
2,500	320	109	1	18
75,000	584	108	1	21

purity are summarized. The solution with an A_P of 21,000 was an enzyme solution prepared by fractional ammonium sulfate precipitation, as described above.

If an enzyme solution purified in this way is centrifuged at high speed, the enzyme is found in the pellet. One of the solutions obtained from a pellet suspension after ultracentrifugation at 48,000 R.P.M. was capable of splitting 525 gm. of acetylcholine per cc. per hour and had a protein content of only 6 to 7 mg. per cc., the A_P thus being at least 75,000. Since this solution appeared to have only one component in an analytical ultracentrifuge run, it seems quite probable that this is virtually the pure enzyme and that the pattern found is consequently that of the pure cholinesterase of the material used. As can be seen from Table II, all the solutions tested have a pattern virtually identical with that of the homogenized suspension. Consequently, the pattern found is that of the pure cholinesterase of the material used.

DISCUSSION

The results described show that a high degree of purity of cholinesterase may be easily obtained by fractional ammonium sulfate precipitation once the mucin present in the electric tissue has been removed. A great part of the protein which is not cholinesterase protein may be precipitated by the ammonium sulfate up to a concentration of 21 per cent, whereas most of the enzyme protein remains in solution. Thereafter, ammonium sulfate at concentrations up to 27 per cent precipitates practically all of the cholinesterase protein, leaving the other proteins in solution. If the original solution obtained after the removal of the mucin is precipitated with ammonium sulfate at 21 per cent, a great part of cholinesterase is precipitated simultaneously and would be lost. Also, if the original solution is precipitated by ammonium sulfate at a concentration of 27 per cent, many other proteins would be precipitated simultaneously and the degree of purity obtained would be much lower. Moreover, part of the enzyme remains in solution and would be lost. The purification has therefore been carried out in several steps in order to avoid the large losses incurred by immediate precipitation at these concentrations.

The precipitation with 40 per cent of ammonium sulfate and filtration of the precipitate in the first step were applied only after it had been repeatedly found that centrifugation of the precipitate at this or slightly lower concentrations of ammonium sulfate was very difficult with the centrifuge available. It is possible that with a more efficient centrifuge than that used it would be feasible to separate the precipitate from the supernatant fluid.

It is also important to observe the slight acidification described (pH 5.6 to 5.9). At this pH precipitations are more complete. Stronger acidification, however, should be avoided, since the enzyme is apparently sensitive to greater pH changes.

In a freshly prepared homogenized suspension of electric tissue of *Electrophorus electricus*, 1 mg. of protein splits about 50 to 100 mg. of acetylcholine per hour (A_P = 50 to 100). Since the A_P rises up to more than 20,000 by fractional ammonium sulfate precipitation, a 200- to 400-fold purification has been obtained. An A_P of 20,000 means that in spite of the extraordinarily high concentration of cholinesterase in electric tissue and in spite of its low protein content, the enzyme forms less than 0.5 per cent of the tissue protein. But even this figure is much too high. By high speed centrifugation, the enzyme protein may be further separated from other proteins, indicating that the enzyme protein forms less than 1 per thousand of the total.

Since the amount of enzyme protein is so small in the electric organ,

the high speed of acetylcholine hydrolysis suggests the possibility of a great efficiency of the enzyme system.   Experiments with high speed centrifugation carried out in collaboration with Dr. Kurt G. Stern, which will be described in a separate paper, indicate that the molecular weight of the enzyme is in the neighborhood of 3 million and that its turnover number is approximately 20 million per minute.   Thus, 1 enzyme molecule seems to be able to split 1 molecule of acetylcholine in 3 to 4 millionths of a second.   If these results can be confirmed by the investigations still in progress, they would be noteworthy not only from a purely biochemical view.   In the face of the assumption that the release and removal of acetylcholine are associated directly with the electrical manifestations of the surface membrane in nerve and muscle, the high potential rate of acetylcholine inactivation is of considerable physiological interest.

SUMMARY

A method is described by which a highly purified solution of cholinesterase is obtained from the electric organ of *Electrophorus electricus*. The mucin present in the organ is removed and fractional ammonium sulfate precipitation is used in carrying out the separations.

In a concentration of ammonium sulfate up to 21 per cent, the enzyme protein remains in solution, whereas many other proteins are precipitated and may thus be removed.   By precipitation with ammonium sulfate at a concentration of 27 per cent, the cholinesterase is nearly completely precipitated, whereas other proteins remain in solution at that concentration. In this way, an enzyme solution may be obtained in which 1 mg. of protein splits 20,000 to 21,000 mg. of acetylcholine per hour.   Since in a freshly homogenized suspension of the tissue used 1 mg. of protein splits 50 to 100 mg. of acetylcholine, the preparation obtained represents a 200- to 400-fold purification.   The precipitations with ammonium sulfate have to be carried out in several steps.

A further separation of inactive protein from the enzyme protein may be obtained by high speed centrifugation.

The pattern obtained by testing the rate of hydrolysis of different esters is typical for cholinesterase and remains unchanged throughout the whole process of purification.   It is still the same if a higher degree of purification is obtained by high speed centrifugation.   Thus, the esterase present in electric tissue seems to be exclusively cholinesterase.

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THE PHOSPHORUS-CONTAINING LIPIDES OF THE CARROT*

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The early literature dealing with plant phospholipides was reviewed in 1930 by Thierfelder and Klenk (1) and, with the exception of a few of the most recent papers (2–5), the work in this field was summarized in 1941 by Working and Andrews (6). The difficulties in isolating and purifying plant phospholipides and in freeing them of sugars, amino acids, etc., are brought out in these reviews. Such difficulties are particularly evident in plants like the carrot (7–13) that are high in water and low in lipide content.

The present report deals with the chemical characteristics of the phosphorus-containing lipides of the carrot. Two types are described, differing primarily in their choline and nitrogen contents. One type, obtained from the raw carrot, was low in nitrogen and low in, or devoid of, choline. The other, obtained from steam-treated carrots, was higher in its nitrogen and choline content.

EXPERIMENTAL

Isolation of Phospholipides from Carrot

The carrots used throughout were obtained from the University farm at Davis[1] or were purchased in the open market. They were cleaned, scraped, and ground. The ground material was then treated with various solvents, as described below.

Two extraction procedures were used; these differed essentially in the temperature at which the extraction was carried out and in the type of solvent employed. In the first the raw carrots were extracted with an alcohol-ether mixture at 55–60°. In the second a slush was first made of ground carrots with ethyl ether and the mixture was frozen at −25°. This served to rupture the cell membranes, the lipides freed by this procedure being dissolved in ethyl ether.

1. Extraction of Raw Carrot at 55–60°—15 kilos of fresh carrots were transferred in portions to round bottom flasks. A sufficient amount of a 3:1 alcohol-ether mixture was added to give a ratio of 3 cc. of solvent per

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gm. of carrot.   The mixture was then refluxed for 18 to 24 hours at 55–60°.
The solvent was decanted and filtered.   The carrot residue obtained after
this extraction was subjected to another treatment with 3:1 alcohol-ether
and both extracts combined.   It was then concentrated at 55–60° to a
small volume (about 10 to 100 cc., depending on the water content) under
reduced pressure (40 to 60 mm. of Hg) in an atmosphere of CO_2.   The
concentrated material was then reextracted with several volumes of distilled
petroleum ether (b.p. 30–60°) and the combined petroleum ether extracts
reduced to a volume of about 10 cc. under reduced pressure in an atmos-
phere of CO_2.   To the concentrate so obtained 2 to 3 volumes of acetone
were then added, and this was followed by the addition of several drops of a
saturated solution of $MgCl_2$ in absolute alcohol.   The precipitate that
formed was washed repeatedly with acetone and then dissolved in a small
volume of moist freshly distilled ethyl ether.   The precipitation procedure
with acetone and $MgCl_2$ was repeated and the precipitate obtained was
again dissolved in moist ethyl ether.

    2. Extraction of Raw Carrots at Low Temperature—5 kilos of fresh raw
carrots were ground and portions transferred to a round bottom flask.   A
sufficient amount of freshly distilled ethyl ether was added to give a ratio
of 3 cc. of solvent per gm. of carrots and the slush intimately mixed by
swirling.   It was then frozen by immersion in an alcohol-solid CO_2 bath
(below −25°).   The frozen mass plus ether supernatant was then placed
in a 5° room for 6 hours and allowed to melt.   At the end of this period the
ether supernatant was decanted, fresh ether was added to the residue, and
the freezing procedure was repeated.   The combined ether extracts were
then concentrated and the residue taken up in petroleum ether.   The
isolation of the phospholipides by precipitation with acetone and $MgCl_2$, as
described in Section 1 preceding, was then carried out.

    3. Extraction of Steam-Treated, Dehydrated Carrots at 55–60°—Freshly
scraped carrots were cut into slices ½ inch thick, placed on wire trays, and
immediately thereafter exposed in a preheated steam box to live steam for
5 minutes.   The slices were then dehydrated at 60° for 10 to 12 hours.   The
final material contained less than 10 per cent water.

    In order to extract lipides from the dehydrated carrots, it was found
necessary to rehydrate them.   When the dried material was refluxed
with alcohol-ether mixtures, ethyl ether, or petroleum ether, no measurable
amounts of a lipide substance were extracted.

    The dehydrated material was mixed with enough distilled water to
bring its water content to 20 per cent.   The uptake of water was complete
in 2 to 3 hours.   The rehydrated carrots were first finely ground and then
extracted four times with a 3:1 alcohol-ether mixture.   The extracts were
combined and the phospholipides isolated after the manner described in
Section 1.

4. Extraction of Steam-Treated Carrots at 55–60°—Freshly scraped carrots were sliced and steam treated for 5 minutes. As soon as the slices were cool enough to handle, they were finely ground and extracted exactly as described in Section 1.

Test of Extraction Procedure

In order to determine the extent to which the phosphorus-containing

TABLE I

Extraction of Phosphorus-Containing Compounds from Raw and Steam-Treated, Dehydrated Carrots

Fraction No.	Extraction No.	Alcohol-ether-soluble P		Petroleum ether-soluble P		
			Total P*		Total petroleum ether-soluble P	Total P*
		mg.	*per cent*	*mg.*	*per cent*	*per cent*
I 1000 gm , raw	I	540	85	20	59	3.0
	II	90	14	12	35	2.0
	III	4	1	2	6	0.3
II. 250 gm. steam-treated, dehydrated	I	270	64	15	25	4.0
	II	70	17	4	7	1.0
	III†	14	3	4	7	1.0
	IV	22	5	14	23	3.0
	V‡	34	8	22	37	5.0
	VI	10	2	1	2	0.5

* The total phosphorus of the carrot was found to be alcohol-ether-soluble. The residue obtained after the last extraction contained no significant amounts of phosphorus.

† Carrot residue from Extraction III rehydrated to 20 per cent water, then extracted

‡ Carrot residue from Extraction IV was ground and then extracted.

lipides can be removed from the raw and dehydrated carrots, the following tests were carried out.

Three successive alcohol-ether (3:1) extracts of the raw carrots were prepared after the manner described in Section 1 above. The total phosphorus content and the portion of this phosphorus soluble in petroleum ether were determined in each of these three extracts. Phosphorus was determined by King's method (14). The results are recorded in Table I.

The raw carrots used here contained phosphorus to the extent of 0.65 per cent of their dry weight, 99 per cent of which was soluble in a 3:1 alcohol-ether mixture. The residue obtained after the three successive extractions described above contained about 0.5 per cent of the initial amount of phosphorus present in the raw carrot. In addition, saponifi-

cation of this residue yielded less than 0.05 per cent "lipide" material. These results leave little doubt of the completeness of the extraction procedure used for the raw carrot.

A small amount of the raw carrot phosphorus extracted by a 3:1 alcohol-ether mixture is soluble in petroleum ether. It amounted to about 5 per cent of the total phosphorus contained in the carrot or about 0.05 per cent of the dry weight of the carrot.

Since it could not be assumed that heat treatment had not altered the solubility properties of the carrot lipides, the extraction procedure was also tested in the steam-treated dehydrated carrots. Dehydrated carrot chips were rehydrated to bring their water content to about 20 per cent, and then extracted with 3:1 alcohol-ether. Six successive alcohol-ether extractions were made, as described above, each succeeding extraction being made on the carrot residue from the previous extraction. These alcohol-ether extracts have been designated Extractions I to VI in Table I. After Extraction III, the residue was rehydrated to bring its water content to 20 per cent and the extraction continued. The residue from Extraction IV was ground before proceeding with the next extraction. The phosphorus content was determined on each alcohol-ether extract and on each petroleum ether extract prepared from them. The values are recorded in Table I.

The results obtained show that the extraction procedure used for the steam-treated dehydrated carrot is satisfactory for the removal of phosphorus-containing lipides. The residue that remained after six alcohol-ether extractions contained about 2 per cent of the total phosphorus present in the fresh carrot. Less than 0.1 per cent saponifiable material was found in this residue, and this consisted largely of coloring matter.

It is again evident that the phosphorus of the carrot soluble in petroleum ether is small in amount. About 0.5 per cent of the dry weight of the carrot was phosphorus, which was completely soluble in 3:1 alcohol-ether. But the phosphorus soluble in petroleum ether amounted to only about 0.07 per cent of its dry weight.

The amounts of petroleum ether-soluble, acetone-insoluble phosphorus (*i.e.*, phospholipide) found in the various batches studied are recorded in Table II. The percentages varied from 0.1 to 0.6 per cent of the dry weight of the carrot.

Characteristics and Composition of Petroleum Ether-Soluble, Acetone-Insoluble Lipides of Carrot

More than 90 per cent of the petroleum ether-soluble phosphorus obtained from both raw and dehydrated carrots was precipitated by acetone and $MgCl_2$. Four reprecipitations failed to alter its phosphorus content.

The isolated phospholipide was an amorphous, almost pasty substance that was quite soluble in petroleum ether, ethyl ether, and chloroform; it was insoluble in acetone and only slightly soluble in absolute or 95 per cent alcohol. The phospholipide obtained from the dehydrated carrot was readily emulsifiable by water; that obtained from the raw carrot was not.

The composition of the various phospholipides extracted from carrots is shown in Table III. Choline was determined by Glick's method (15), total nitrogen by the micro-Kjeldahl method, and iodine numbers by the Hanus method (16). The total glycerophosphoric acid content and the amounts of its α and β isomers were determined by Burmaster's procedure (17). The fatty acid content was obtained as follows: the sample was saponified with 0.5 N alcoholic alkali, the unsaponifiable matter extracted with ethyl or petroleum ether, and the soaps acidified and extracted with

TABLE II

Content of Phosphorus-Containing Lipides in Raw and Steam-Treated Carrots.
All values are expressed as per cent of dry weight.

Raw carrot					Steam-treated carrot	Steam-treated, dehydrated carrot
Low temperature extraction		Extraction at 55–60°			Extraction at 55–60°	Extraction at 55–60°
Batch A	Batch B	Batch C	Batch D	Batch E	Batch E	Batch F
0 2	0.2	0 5	0.4	0.5	0 6	0.4

ethyl or petroleum ether; the fatty acids were then dried at 70° *in vacuo* for 1 to 2 hours and weighed.

The difference in the nitrogen content of the raw and steam-treated material was not anticipated but, as may be deduced from Table III, can be accounted for, within 10 per cent, by the difference in the choline contents of the two fractions. The phosphorus-containing lipides obtained from the raw carrots were devoid of, or low in, choline. As was to be expected, therefore, the molal ratios of nitrogen to phosphorus were much lower for the phosphorus-containing lipides obtained from the raw than from the dehydrated carrot. As shown by a study of iodine numbers, dehydration of the carrots before their extraction reduced the unsaturated fatty acid content of the phospholipides. The degree to which the raw carrot was divided before its extraction did not influence the composition of the phosphorus-containing lipides isolated.

It was noted that a sugary material was associated with the phospholipide fraction of the dehydrated carrot which was not observed in the raw carrot. This sugary material was intimately associated with the fatty acid fraction

of the phospholipide and was extracted with the fatty acids. So far it has not been possible to purify this substance sufficiently for identification. The impure material, however, gave qualitative tests (Molisch, Fehling) for a carbohydrate. It also formed an osazone, which had a crystalline form similar to glucosazone. Owing to difficulty in purification of the small quantity of osazone involved, a satisfactory melting point was not obtained.

TABLE III

Composition of Phosphorus-Containing Lipides Isolated from Raw and Steam-Treated Carrot

Constituent	Raw carrot					Steam-treated carrot	Steam-treated, dehydrated carrot
	Low temperature extraction		Extraction at 55–60°			Extraction at 55–60°	Extraction at 55–60°
	Batch A	Batch B	Batch C	Batch D	Batch E*	Batch E*	Batch F
Fatty acid, %	58	59	60	58	66	63	59
Phosphorus, %	4.4	5.2	3.5	3.0	2.7	3.2	2.8
Nitrogen, %	0.43	0.33	0.64	0.42	0.72	1.3	1.1
Choline, %	0	0	0	0	0.52	4.4	4.2
N:P, molal ratio	0.21	0.14	0.40	0.31	0.58	0.89	0 89
Choline:P, molal ratio	0	0	0	0	0.05	0.35	0.38
Glycerophosphoric acid, %			11.6	11.0	15 3	17.0	13.5
α-, %			35	34			44
β-, %			65	66			56
Iodine No.	86.0	90.0	196	144	59	54	38

* These represent a uniform lot of carrots that were analyzed at the same time by these two procedures.

DISCUSSION

It is significant that the concentration of phosphorus, nitrogen, and fatty acids is lower in the petroleum ether-soluble, acetone-insoluble fraction prepared from carrots than in identically prepared fractions (phospholipides) isolated from animal tissues.[2] Since the petroleum ether-soluble, acetone-insoluble fraction isolated from steam-treated carrots contains fatty acids, glycerophosphoric acid, and choline, and since, moreover, its molal ratio of nitrogen to phosphorus is close to unity, this fraction may also be considered a phospholipide. The sugary material found associated with the phospholipide of the dehydrated carrot either as a contaminant or as an integral part of the molecule accounts, in part at least, for its low content of nitrogen, phosphorus, and fatty acids.

[2] Phospholipides obtained from animal tissues contain about 4 per cent phosphorus, 2.2 per cent nitrogen, and about 70 per cent fatty acids.

Judging from the molal ratios of nitrogen to phosphorus, or of choline to phosphorus, recorded in Table III, the phosphorus-containing lipide isolated from raw carrots is not a typical lecithin or cephalin or sphingomyelin or a mixture of these three phospholipides. The low nitrogen content and in particular the low content of choline in the fraction obtained from raw carrots suggest its similarity to compounds like the phosphatidic acids which have been obtained from oat seeds by Trier (18) and from cabbage leaves by Chibnall and Channon (19). The presence of small amounts of phosphatidic acids might explain the observed molal ratios of nitrogen to phosphorus of 0.89 for the fractions obtained from steam-treated carrots.

In attempting to explain the isolation of two distinct types of phospholipides from the carrot, it is necessary to point out that the phospholipide with the higher choline content was isolated from carrots that had been exposed to the temperature of live steam before they were subjected to grinding and prolonged treatment with solvents at 55–60°. It is therefore reasonable to assume that the one with the higher choline content is the naturally occurring phospholipide of the carrot and that enzymatic separation of its choline during the grinding and prolonged extraction procedure accounts for the phospholipide being devoid of, or low in, choline. Hence it is postulated that there exists in the raw carrot an enzyme, probably a lecithinase, capable of splitting choline from phospholipides.

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SUMMARY

1. The isolation, characteristics, and composition of phosphorus-containing lipides from raw and steam-treated carrots are described. It is shown that phosphorus-containing lipides can be completely extracted from carrots with alcohol-ether mixtures.

2. The phosphorus-containing lipide isolated from the raw carrot was characterized by a low nitrogen content and by an absence or low content of choline; that isolated from the steam-treated carrot was higher in its choline and nitrogen content.

3. An explanation of the isolation of the two types of phosphorus-containing lipides from carrots is presented. It is postulated that the raw carrot contains an enzyme, probably a lecithinase, able to split choline from phospholipides.

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ALLOXAN STUDIES: THE ACTION OF ALLOXAN HOMOLOGUES AND RELATED COMPOUNDS

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The mechanism by which alloxan destroys the insulin-producing pancreatic islet cells, thus causing diabetes, has so far remained obscure. One approach to this question is the determination of the chemical specificity of the effect. Earlier studies (1, 2), involving a small number of rather randomly selected compounds, failed to disclose any new active agents. 2 years ago, an investigation of a larger series of alloxan homologues and related compounds was begun at this laboratory. It could be shown (3) that, in addition to alloxan, methylalloxan and the corresponding alloxantins and dialuric acids are diabetogenic; some of these findings have since been confirmed independently by Laszt (4) and Hidy (5). The full report of our study which now comprises twenty-nine compounds, among them a number of new alloxan homologues, is herewith submitted, together with an investigation of the possible rôle in the production of diabetes of two characteristic reactions of alloxan, amino acid and glutathione oxidation.

EXPERIMENTAL

Methods

Preparation of Compounds—Previously published methods have been followed as indicated, sometimes with slight modifications. The purity of the preparations was checked by their melting points, and in some cases also by nitrogen analysis. The synthesis of the new compounds propyl-, butyl-, isobutyl-, phenyl-, and benzylalloxan and of their intermediates will be described elsewhere.[1]

Testing of Compounds—Male white rats, weighing between 80 and 130 gm. were used; they were fed on whole wheat with ample additions of vegetables and occasional supplement of bread and bran. The substances tested were injected intravenously, usually in the form of a freshly prepared 1 per cent solution. On the basis of experience with nearly 400 alloxan-treated rats, the following method of interpretation of the results has been adopted. A rat was considered to be strongly diabetic if full glucosuria (more than 1 per cent glucose) occurred within 24 hours after in-

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[1] Brückmann, G., and Isaacs, S. D., to be published.

jection and persisted for at least 3 days. Rats in which the onset of glucosuria was delayed (sometimes up to 5 days) or in which it subsided within less than 3 days were regarded as slightly diabetic. Only seldom were dubious cases seen, *i.e.* rats which excreted less than 1 per cent glucose for 1 or 2 days; these are discussed individually. When necessary, additional information was obtained by observation of blood sugar (Somogyi (6)), urine ketone, urine volume, body weight, and temperature.

As a quantitative measure of the potency of some of the diabetogenic compounds the dose which caused diabetes in 50 per cent of the animals (effective dose, ED_{50}) was calculated according to established statistical methods (7). Details of this calculation will be reported separately.

Results

Alloxan and Mono-N-Substituted Alloxans,[2]

$$\begin{array}{cc} RN\text{—}CO \\ | \qquad | \\ OC \quad CO \\ | \qquad | \\ HN\text{—}CO \end{array}$$
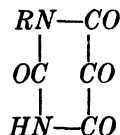
Alloxan; Prepared from Uric Acid (8)—The ED_{50}, as determined in 125 rats, was 45 mg. (0.28 mM) per kilo, with the lowest observed active dose of 30 mg. per kilo. Signs of acute toxicity, as described in detail under ethylalloxan, appeared following injection of 150 or more mg. per kilo. Doses above 350 mg. per kilo proved lethal within 4 hours. Severe uremia and occasionally albuminuria were observed with 70 mg. per kilo and above.

Methylalloxan; Prepared from Theobromine (9)—The ED_{50} in 51 rats was 53 mg. (0.30 mM) per kilo, and the lowest observed active dose 40 mg. per kilo. As with alloxan, severe cases of diabetes with extreme hyperglycemia, ketonuria, and death in diabetic coma were encountered, but the lower doses caused a higher incidence of transitory diabetes (less than 3 days duration) as compared with alloxan.[3] Early toxic symptoms first began to appear with 70 mg. per kilo; doses over 200 mg. per kilo caused death within 4 hours. Uremia and albuminuria were observed in many cases.

Ethylalloxan; Prepared according to Bülmann and Klit (10) and Biltz and Sedlatschek (11)—Of forty rats, injected with 50 to 130 mg. per kilo, fourteen became diabetic. The ED_{50} was 67 mg. (0.355 mM) per kilo, the lowest observed active dose 60 mg. per kilo. Early signs of acute toxicity appeared at a dose of 50 mg. per kilo, as follows:

About 1 minute after injection, usually after a transient excitation period, a characteristic syndrome with lacrimation, salivation, nasal discharge,

[2] In alloxan the R in the formula is H.
[3] Brückmann, G., to be published.

heavy respiration, and frequent gasping for air becomes apparent. During the next minutes the symptoms increase in severity, the animal lying on its side with closed eyes and appearing ill and weak. Defecation and a slowed heart rate are frequent. In recovery, the symptoms subside within an hour or so; if no recovery is made, death with signs of cyanosis and asphyxia ensues. In postmortem examinations of animals which died within 24 hours after injection, profuse pulmonary edema and severe hemorrhage in the lungs were found in almost every case. It would appear, therefore, that damage to the lung capillaries accounts largely for the syndrome so far described. Other macroscopic observations are general cyanosis, distended stomach, and the presence of bilirubin in the intestines, which are frequently yellow in color.

The same acute symptoms, in varying degrees of intensity, were observed with all members of the alloxan series and will be referred to subsequently as "early symptoms."

The higher doses usually caused death before severe diabetes could develop, so that on the whole ethylalloxan diabetes appeared milder than that produced by alloxan. Kidney damage, too, seemed to be less pronounced than with alloxan.

Propylalloxan[1]—Preliminary trials with this substance showed it to be highly toxic, and it seemed unlikely that a diabetogenic dose would be survived. Severe early symptoms were seen with 45 mg. per kilo; 70 mg. caused death within 24 hours. Use was therefore made of the recently discovered, apparently specific, potentiating action of ammonium salts on the diabetogenic action of alloxan. It had been found by us[3] that simultaneous injection of alloxan with ammonium salts decreased the ED_{50} to about 50 per cent. Consequently, thirteen of the fifteen rats which were injected with doses of propylalloxan ranging from 45 to 70 mg. per kilo received simultaneously 100 mg. per kilo of ammonium chloride. By this device it became possible to produce diabetes in three rats, as follows: Rat 1, glucosuria (about 2 per cent); blood sugar 490 mg. per cent; died 2 days after injection. Rat 2, observed 15 days; slight transient diabetes; little urine voided; 1.5 per cent glucose in one sample; blood sugar 276, 215, and 160 mg. per cent on the 2nd, 4th, and 5th days respectively; thereafter normal; glucose tolerance on the 3rd day decreased. Rat 3, observed 20 days; 10 days heavy glucosuria with polyuria, but no ketonuria; blood sugar 200 and 430 mg. per cent; diabetes subsided thereafter. Albuminuria and moderate uremia were observed in several rats.

Butylalloxan[1]—The toxicity of this compound seemed to be even higher than that of propylalloxan, and ammonium chloride was therefore again used in order to potentiate any possible diabetogenic effect. Twenty-three rats

received doses from 45 to 78 mg. per kilo, mostly with ammonium chloride. Eleven survived for more than 1 day, but none developed clear cut diabetes. One died after 24 hours with a terminal blood sugar of 300 mg. per cent and slight glucosuria. Terminal blood sugar values must, however, be interpreted with great caution, since they are frequently elevated by unspecific factors. One rat survived over 15 days and excreted during the first 3 days urine with small amounts (0.5 to 1.0 per cent) of glucose. This case is suspicious of slight diabetes. Uremia, albuminuria, and anuria were observed.

Isobutylalloxan[1]—The iso compound appeared to be somewhat less toxic than the *n* isomer, but again no clear cut diabetes could be produced. Nine rats were given 45 to 100 mg. per kilo; three of them received simultaneously ammonium chloride. Six survived for over 1 day. One had a blood sugar (single examination) of 200 mg. per cent, but no glucosuria, another slight glucosuria (0.5 to 1.0 per cent) for 2 days, and 200 mg. per cent of blood sugar. As in the case of butylalloxan, the possibility exists that this compound causes slight impairment of carbohydrate metabolism, but no complete necrosis of the islet cells which would result in the development of true diabetes.

Phenylalloxan[1]—By introduction of the phenyl group a practically nontoxic compound was obtained, which was even less toxic than alloxan itself. Early symptoms appeared only with 300 mg. per kilo, and doses up to 500 mg. per kilo were tolerated for more than 1 day. Of thirteen rats which received between 120 and 600 mg. per kilo (together with ammonium chloride in seven) none showed signs of diabetes, but a few had slight uremia.

Benzylalloxan[1]—This substance was about as toxic as ethylalloxan. Thirteen of nineteen animals injected with 50 to 100 mg. per kilo (part of them with NH_4Cl) survived for more than 24 hours. As in the case of phenylalloxan, no dubious diabetes was encountered; therefore it seems safe to conclude that this compound is non-diabetogenic in the dosages tried. Moderate uremia and albuminuria were again present.

$$\text{Di-N-Substituted Alloxans,} \quad \begin{array}{c} RN-CO \\ | \quad | \\ OC \quad CO \\ | \quad | \\ RN-CO \end{array}$$
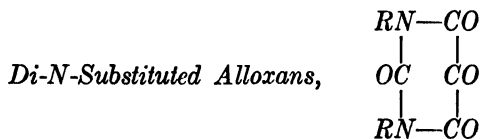
Dimethylalloxan; Prepared from Caffeine (9)—Thirty rats received from 40 to 250 mg. per kilo; a few of them ammonium chloride, simultaneously. As already reported (3), the compound is very toxic; initial symptoms appeared with the lowest doses, and only ten of the animals survived doses up

to 80 mg. per kilo for more than 1 day. None of them showed signs of diabetes. Kidney damage, on the other hand, seemed to be more pronounced than with the monosubstituted alloxans, since each of six rats examined had severe uremia and frequent albuminuria. Dimethylalloxan is non-diabetogenic also by the subcutaneous route, as has recently been reported by Hidy (5). We found the substance also very toxic for cats, rabbits, and mice.

Methylethylalloxan; Prepared according to Biilmann and Berg (12)—Of nine rats injected with 50 to 90 mg. per kilo, together with ammonium chloride, only two survived; they showed no signs of diabetes. The early symptoms and kidney damage were similar to those observed with dimethylalloxan.

Methylpropylalloxan—This was prepared from the previously known dimethyldipropylalloxantin (13) by oxidation with nitric acid; m.p. 92°, easily soluble in water. On injection it proved to be the most toxic of this group. Of eleven rats given doses up to 60 mg. per kilo (with NH_4Cl) five survived, but did not become diabetic. As in the case of the other disubstituted alloxans, severe uremia developed in rats which died more than 24 hours after injection.

Dialuric Acids,[4]

$$
\begin{array}{c}
RN\!-\!CO \\
| \qquad | \quad\diagup H \\
OC \quad\ C\!\diagdown \\
| \qquad | \quad OH \\
RN\!-\!CO
\end{array}
$$
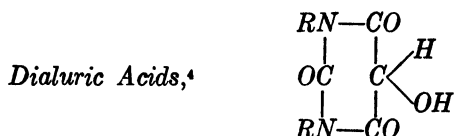
Dialuric Acid; Prepared according to Biltz and Damm (14)—As already reported (3), we had no difficulty in eliciting diabetes with this substance at dosages from 50 to 150 mg. per kilo. In the first experiments solutions were prepared simply by heating the substance in water. Attention has been drawn, however, to the high autoxidizability of dialuric acid (15), and Lazarow has expressed the view that our positive results might have been due to the presence of alloxan formed by autoxidation of dialuric acid (16). We have therefore repeated the tests, using this time air-free water under an atmosphere of CO_2, as advocated by Archibald (15). In addition, dialuric acid and alloxan were determined in the solutions by Archibald's method (reduction of phosphotungstic acid without and with the addition of potassium cyanide) before and after injection. In four of six rats given doses of 60 to 70 mg. per kilo typical diabetes, sometimes with ketonuria, developed. Since the alloxan content of the solutions corresponded to only about 20 mg. per kilo, an amount which does not in itself cause diabetes, there can be no doubt that the active agent is dialuric acid. Dialuric acid

[4] In dialuric acid the two R groups are hydrogen atoms.

diabetes has also been produced recently by Laszt (4); previous workers, on the other hand, reported negative results with this substance (1, 2).

The following observation makes it seem probable that injected dialuric acid is quickly oxidized *in vivo* to alloxan, and suggests that positive tests with this substance do not bear on the specificity of the alloxan effect. Blood drawn immediately after injection appeared very dark, and showed on spectroscopic examination increased amounts of reduced hemoglobin. *In vitro*, the spectrum of oxyhemoglobin disappeared almost instantly when hemolyzed blood was incubated with dialuric acid.

Monomethyldialuric Acid; Prepared according to Biltz and Damm (14)— Since this substance is easily soluble in cold water, the risk of autoxidation is smaller than in the case of dialuric acid. Four rats were injected with doses from 40 to 130 mg. per kilo, and two of them developed typical diabetes. As with dialuric acid, reduction of oxyhemoglobin was observed in blood samples. Again, therefore, the actual diabetogenic agent seems to be formed through oxidation of methyldialuric acid to methylalloxan by blood hemoglobin.

Dimethyldialuric Acid; Prepared according to Biltz and Damm (14)—We failed to isolate the very unstable free acid, and the more stable sodium salt was therefore used. The physiological properties of this substance seem to be identical with those of dimethylalloxan; diabetes was not produced in any of seven rats which received from 50 to 80 mg. per kilo. Again, hemoglobin was readily reduced both *in vitro* and *in vivo*.

$$\textit{Alloxantins,} \qquad \begin{array}{ccccc} (H \text{ or } R)N\text{---}CO & OH & H & CO\text{---}N(H \text{ or } R) \\ | & |\diagup & \diagdown| & | \\ OC & C\text{------}O\text{------}C & CO \\ | & | & | & | \\ HN\text{---}CO & & CO\text{---}NH \end{array}$$
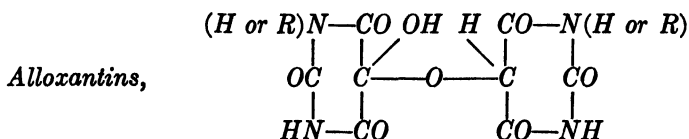
Since alloxantins are known to dissociate in aqueous solutions into alloxans and dialuric acids (17), each diabetogenic, it is to be expected that these substances will have the same effect as the alloxans. This proved to be the case with alloxantin, dimethyl- and diethylalloxantin, which appeared physiologically identical with the corresponding alloxans, and like the latter produced diabetes. Therefore details need not be given; the methods of preparation have been cited (see the corresponding alloxans). Goldner and Gomori (1) reported alloxantin to be inactive, but Koref *et al.* (18) found it to be diabetogenic in rabbits.

Substitutions in C_5 Position in Alloxan Molecule

The most reactive group in the molecule is the central $\overset{|}{\underset{|}{C}}O$ group, or

more correctly its hydrated form $\overset{\displaystyle OH}{\underset{\displaystyle OH}{C{<}}}$. Results of previous investiga-

tions have indicated that substitution in this position abolishes the physio-

logical activity of alloxan. Barbituric acid, CH_2, violuric acid, $C{=}NOH$,

and uramil, $\overset{\displaystyle H}{\underset{\displaystyle NH_2}{C{<}}}$, have been found inactive (1,2). We have now confirmed

these findings by intravenous tests on rats. Since uramil is only sparingly soluble, the more soluble N-methyluramil (14) was tried, but gave no better results. Even a minor modification of the structure, such as the esterification of one OH group, as in benzoyl-N-methyldialuric acid
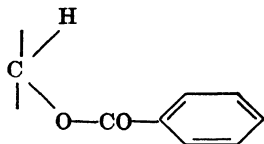
prepared according to Nightingale (19), rendered the compound inactive.

Compounds Giving Strecker Reaction

Ability to decarboxylate and desaminate α-amino acids, with subsequent formation of an aldehyde with 1 less carbon atom, is one of the better known properties of alloxan. This reaction, which is named after its discoverer Strecker (20), is also given by a number of compounds which are structurally related to alloxan. Investigation of this group was therefore deemed of interest.

Ninhydrin, CO—Fourteen rats were injected with doses

ranging from 30 to 120 mg. per kilo. The high acute toxicity of ninhydrin, which had been observed by its discoverer Ruhemann (21), could be lowered by slowing up the injection rate. Doses up to 60 mg. per kilo, given in this way, were tolerated for more than 2 days. The early symptoms resembled those produced by the more toxic alloxans, and pulmonary edema was found at autopsy; kidney damage, however, as judged by albuminuria and uremia, was less pronounced than in the alloxan series. As has already been reported (3), ninhydrin did not cause diabetes. Hidy (5),

who administered the substance intraperitoneally, has recently confirmed this finding.
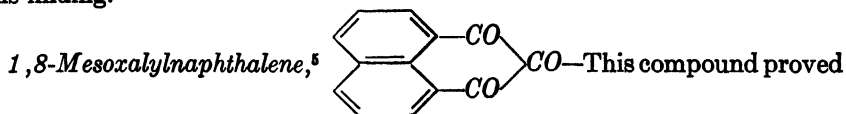
1,8-Mesoxalylnaphthalene,[5] —This compound proved less toxic than ninhydrin, but was slightly soluble, and no more than 50 mg. per kilo could be injected. The substance gave a very intense Strecker reaction, perhaps more so even than ninhydrin, but failed to produce diabetes, even on repeated injection.
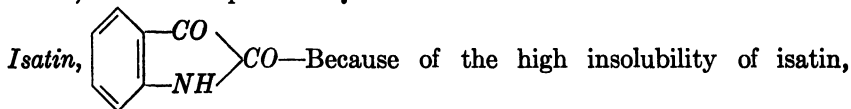
Isatin, —Because of the high insolubility of isatin, only a few injections with oversaturated solutions succeeded. Doses of 50 to 80 mg. per kilo produced neither diabetes nor toxic symptoms.

1,2-Naphthoquinone-4-sulfonic Acid (Sodium Salt),
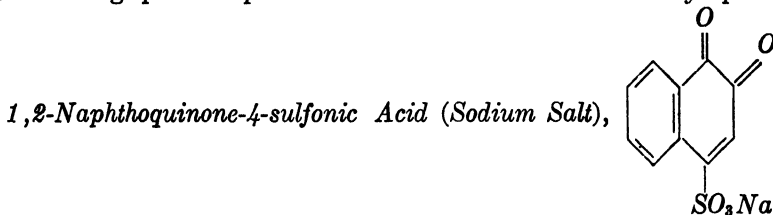
This was the only substance outside the alloxan series which produced some disturbance in carbohydrate metabolism. Unfortunately, the toxicity of the compound is considerable, and doses higher than 150 mg. per kilo were not tolerated for more than 1 day. Lung and kidney damage, as judged by the presence of pulmonary edema and uremia, were prominent. Twenty-nine rats were injected with from 80 to 180 mg. per kilo; sixteen of them received ammonium chloride simultaneously. Of the entire group, twenty survived the 1st day. The chief early symptom was respiratory trouble, associated with severe cyanosis. The blood became dark brown and remained so for days. Spectroscopic examination showed the presence of methemoglobin; this pigment was also produced *in vitro* upon addition of the drug to blood.

While no case of clear cut diabetes was encountered, a number of rats showed signs of impaired carbohydrate metabolism. Rat 25 on the 2nd day voided urine with 1.5 per cent glucose, then for a few days urine with traces of glucose, and afterwards became normal. Rat 17 showed on the 3rd day a blood sugar of 307 mg. per cent, but no glucosuria. Intravenous glucose tolerance was normal. Rat 18 excreted urine containing about 0.5 per cent glucose and showed on the 3rd day a blood sugar level of 248 mg.

[5] Kindly supplied by Professor A. Schönberg, Fuad I University, Cairo.

per cent. Three other rats excreted sometimes traces of sugar in their urine and died after 2 days with terminal blood sugars of 200, 316, and 560 mg. per cent respectively. The doubtful significance of terminal blood sugar has already been noted. A rabbit received 120 mg. per kilo without development of diabetes within 6 days. We must thus conclude that, while the substance cannot be considered diabetogenic in the doses employed, the possibility exists that it may be potentially diabetogenic with higher doses.

Rhodizonic Acid, —This cyclic tetraketone is listed here
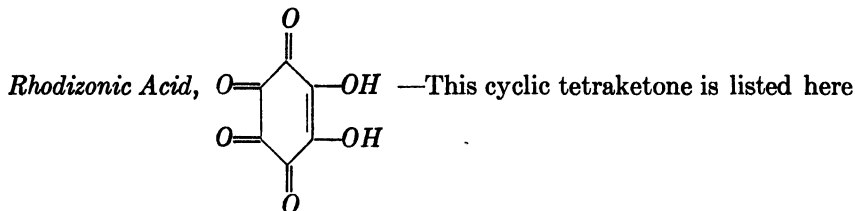
because of its remote structural relationship to alloxan, although it gives no Strecker reaction. Since it proved to be non-toxic, amounts as high as 400 mg. per kilo could be injected without visible effect.

Diabetogenic Action and Strecker Reaction

The results which have been reported so far make it improbable that the islet effect of alloxan can be related to the Strecker reaction. According to determinations of Chargaff and Bendich (22), the potency of ninhydrin is about 8 times and that of 1,2-naphthoquinone-4-sulfonic acid 4 times that of alloxan, as judged by the rate of decomposition of alanine. However, the possibility had to be considered that these compounds fail to cause diabetes because of very rapid destruction in the blood stream. The ninhydrin content of the pancreas was therefore determined by a modification of Brückmann's (23) alloxan method. Half a minute after the injection of 140 mg. per kilo a concentration of about 30 mg. per cent was found in the pancreas. This is even more than the alloxan content of pancreas after alloxan injection.

Another approach to the problem is suggested by the fact that in the alloxan series itself some homologues are diabetogenic, while others are not; this invites a comparison of physiological activity with amino acid oxidation. To measure the latter, the rate of the formation of murexide, an end-product of the interaction of alloxan and amino-acids (24), was followed. This method was used, as the Van Slyke technique (25), for measuring CO_2 as end-product of the reaction of ninhydrin with amino acids, is not applicable to some of the alloxans, for the reason that they decompose with CO_2 formation at 100°. 1 cc. of phosphate buffer of pH 7.2, containing 20 mg. of glycine, was mixed at zero time with 0.1

mM of the various alloxans in 1 cc. of water, and the development of the red murexide color was measured at intervals in the Pulfrich photometer, Filter 470 mμ (Fig. 1). The extinction values were not exactly reproducible from day to day, but the same order of intensities was always obtained with the various compounds; variation of pH or replacement of glycine by alanine did not alter this order. The curves in Fig. 1 are constructed from the average values of three or four sets of experiments. Comparison of the extinction values after 30 minutes reveals the following activities, relative to alloxan as 1.0: butylalloxan 0.81, propylalloxan 0.66, methylalloxan 0.50, ethylalloxan 0.41, benzylalloxan 0.30, and dimethylalloxan 0.18
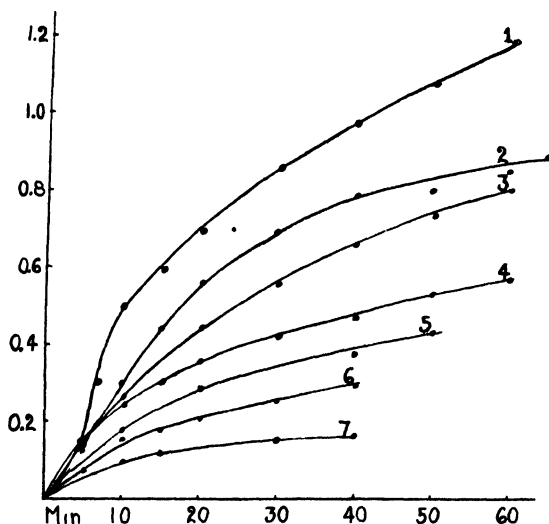
FIG. 1. Murexide formation from glycine (20 mg.) by various alloxans (0 1 mM); pH 7 2 Ordinate scale, extinction (530 mμ) Curve 1, alloxan; Curve 2, butylalloxan; Curve 3, propylalloxan; Curve 4, methylalloxan; Curve 5, ethylalloxan; Curve 6, benzylalloxan; Curve 7, dimethylalloxan.

Phenylalloxan gave practically no murexide color. The physiological activities, calculated from the ED_{50} values reported above, are as follows: alloxan 1.0, methylalloxan 0.93, ethylalloxan 0.79, while propyl- and butylalloxan, which give an intense Strecker reaction, are only weakly diabetogenic or entirely inactive. There seems, therefore, to be no correlation between the Strecker reaction and islet damage.

Two by-products of the Strecker reaction, a derivative of uramil (diuramil) and a "reducing substance" having the elementary formula $C_9H_{11}O_7N_5$, have been isolated by Hurtley and Wootton (24). Both compounds, when tested by us on rats, were found to be inactive. The fact that murexide itself does not cause diabetes has already been reported (3).

Diabetogenic Action and SH Oxidation

Even before the discovery of the diabetogenic action of alloxan, the high affinity of this compound for SH groups was emphasized (26, 27). In view of the demonstration that the mode of action of an increasing number of pharmacologically active substances depends on oxidation of SH groups in enzymatic and non-enzymatic processes, it is not surprising that attempts have been made to explain the alloxan effect on pancreas on this basis. Experimental evidence for this theory has been advanced by Leech and Bailey (28), who observed a rapid decrease in blood glutathione in rabbits after diabetogenic, sometimes smaller, doses of alloxan. The restoration of the glutathione level to normal took several hours. Similar observations were earlier reported, without comment, by Eckert et al. (29), but appear to have been overlooked. Lazarow (16) has recently shown that the diabetogenic effect of alloxan is prevented by a simultaneous injection of 10- to 30-fold amounts of glutathione or cysteine; he does not think that this antagonistic action is due simply to decomposition of alloxan in the blood stream by these substances.

To obtain more evidence on this question, we repeated the experiments of Leech and Bailey (28), using rats. The glutathione content of blood and tissues was determined by a modification[6] of Fujita's (30) method. The occurrence of a sharp drop in blood glutathione 2 to 5 minutes after injection of 60 to 70 mg. per kilo of alloxan was readily confirmed. Contrary to reports by De Caro and Rovida (31), the reduced glutathione content of organs showed no change in the same conditions (see Table I).

[6] For the determination of reduced glutathione in small samples of *blood*, 0.3 to 0.5 cc of freshly drawn blood is placed in a centrifuge tube containing 1 cc. of water and a little saponin. When hemolysis is completed, 2 5 cc of 3 per cent metaphosphoric acid and about 1 5 gm of solid sodium chloride are added, and the mixture shaken and after a few minutes centrifuged. To 2 cc of the filtered supernatant are added 1 cc. of saturated sodium chloride, 0.5 cc of a freshly prepared solution of 0.1 gm. of sodium nitroprusside in 10 cc. of 0 4 per cent ammonium sulfate, and 0.7 cc. of 25 per cent sodium carbonate (10 per cent, aqueous). The mixture is quickly transferred to a photometer cuvette of 0.5 to 1 cm width and read within 1 minute at 500 to 530 mμ. For *tissues*, 0.2 to 0 5 gm. of freshly excised or CO_2-frozen tissue are extracted in a small mortar with 2 5 cc. of 3 per cent metaphosphoric acid, about 1.5 gm. of sodium chloride, and 1 cc. of water The mixture is transferred to a centrifuge tube, and the centrifugate treated as above. A reagent blank is run, in which the 2 cc. of extract are replaced by 2 cc. of 2 per cent metaphosphoric acid saturated with sodium chloride. When *no more than 8 minutes elapse* between the preparation of the nitroprusside solution and the addition of sodium carbonate, this method yields colors of good intensity, stable for at least 1 minute, and little affected by temperature. Beer's law is strictly valid. Speed is unnecessary in the intermediate steps of the method, since metaphosphoric acid extracts can be left for 1 to 2 hours at 4° without loss of glutathione.

CO_2-frozen samples, analyzed immediately after extirpation under urethane narcosis, were used throughout. The figures for normal rats were in good agreement with those reported by other investigators.

The fact that ninhydrin, a non-diabetogenic substance, causes an even

TABLE I

Reduced Glutathione in Normal and in Alloxan- and Ninhydrin-Injected Rats

All values in mg. per 100 gm. Time of sampling after injection, blood 2 to 5 minutes, pancreas 4 to 9 minutes, kidney 9 to 10 minutes, liver 10 to 11 minutes.

Rat No.	Blood		Pancreas	Kidney	Liver
Normal rats					
1	55				118
2	27		60	134	118
3	36		53	112	202
4	52		71	113	225
5	29		56	92	204
6	22		62	95	188
Average..	37		60	109	176
Alloxan-injected; 60–80 mg. per kilo					
	Before	After			
1	29	18	71	140	275
2	59	41	66	124	220
3	43	10	75	84	250
4	29	22	97	75	107
5	54	34	87	107	230
6	47	59	71	135	182
7	50	27	62		
8	41	9			
9	32	5			
Average .	43	25	75	111	210
Ninhydrin-injected; 50–80 mg. per kilo					
1	51	18	64		
2	28	4	82		
3	45	0	82		

more rapid disappearance of reduced glutathione from the blood than alloxan (Table I) throws doubt on the significance of change in blood glutathione as a cause of islet damage. Apart from ninhydrin, two other non-diabetogenic compounds, butyl- and benzylalloxan, were found to produce a sharp drop in blood glutathione.

DISCUSSION

The available evidence shows clearly that an intact pyrimidine nucleus is essential for diabetogenic action. Activity is abolished by substitution of the alloxan molecule at any position elsewhere than in one imino group.[7] But even in this site substitution, if, as in the case of butylalloxan, carried beyond a certain length of side chain, renders the compound inactive. The inactivity of substances like dimethylalloxan, in which both NH groups are substituted, may be due to a lack of an ionizable hydrogen atom in such compounds (Hidy (5)). Since such agents produce pronounced kidney injury, it is clear that islet and kidney damage is not due to the same mechanism.

No systematic study of the toxicology of this series has yet been undertaken. Present comment will be limited, therefore, to the remark that the observed correlation of toxicity with increasing length of side chain accords with common pharmacological experience. Most of the observed early symptoms can be related to respiratory impairment, which in turn is caused by severe damage to the lung capillaries, leading to hemorrhage and edema.[8] It becomes more and more evident that the toxic action of alloxan is not confined to the pancreatic β-cells. Kidney and lung damage are likewise prominent, particularly in the case of the higher alloxan homologues. A number of authors have reported the production of lesions in various other organs by alloxan (32–38).

On the assumption that islet necrosis is a direct effect of alloxan as such, this view being strongly supported by the histological evidence (see the excellent review of Duff (39)), several modes of action for alloxan can be considered.

1. Selective accumulation in toxic amounts of the drug by the β-cells. This possibility will be difficult to prove experimentally, since in mammals the β-cells comprise less than 0.5 per cent of the pancreas. It has been found by us that after alloxan injection the concentration of the latter is lower in pancreas than in liver or kidney; this does not, however, dispose of the possibility that the drug is at a high concentration in the islets themselves.

2. Competition with a structurally similar compound for an enzyme with resultant disorganization of the cell metabolism and eventual disintegration of the cell structure. This is a highly speculative but at present

[7] Substitution in the C_2 position has not yet been tried; we have been unable to synthesize 2-thio- or 2-iminoalloxan (Brückmann and Isaacs, unpublished).

[8] In our first communication (3) we have expressed the view that the initial symptoms might be caused by vagus stimulation. This interpretation is not, however, supported by pharmacological findings which have since been made in this laboratory.

a very popular explanation of drug action.   A substrate similar to alloxan is not known in the mammalian organism.   Moreover, the substitution of a propyl group in the small molecule of alloxan must involve a considerable steric alteration; yet, as shown, the resulting compound is diabetogenic. If the claim of Shaw-Dunn *et al.* (40), that a styrylquinoline has the same effect as alloxan, is corroborated, or if it should be possible to demonstrate, by histological examination, that 1,2-naphthoquinone-4-sulfonic acid causes some islet damage, the competition theory would have to be abandoned.

3. Specific reaction of alloxan in the islet cell with or without a specific accumulation.   Such a reaction would probably consist in the inactivation of an enzyme system, which can be expected to be intimately concerned with insulin synthesis, apparently the only specific function of the β-cells. Lazarow (16) has expressed the view that inactivation of SH groups of enzyme proteins could account for such an effect.   In view of our demonstration of a large glutathione reserve in the pancreas after alloxan injection, there is reason to consider that any oxidized SH group would quickly be regenerated by glutathione.   As is shown by our study of blood glutathione, SH oxidation is a relatively unspecific property, and we shall have to look for more specific reactions to explain the mode of action of alloxan.

As demonstrated above, the Strecker reaction, too, can scarcely be held responsible for that action.   The fact that the Strecker reaction requires many hours for completion, whereas alloxan damage is histologically demonstrable 5 to 15 minutes after injection (39), is an additional argument against that view.

The main difficulty in the elucidation of the mode of action of alloxan lies in our lack of knowledge of the specific biochemical processes of the target tissue, the islet.   It is safe to predict that progress here would also throw light on the mechanism of insulin synthesis

SUMMARY

A number of alloxan homologues and related compounds have been administered by intravenous injection to rats, with the following findings.

1. N-Methyl-, ethyl-, and propylalloxans are diabetogenic, whereas higher N-substituted alloxans are not.

2. Alloxantin, dimethylalloxantin, diethylalloxantin, and dialuric and methyldialuric acids are likewise able to cause diabetes.   These substances seem to be rapidly oxidized in the blood to corresponding alloxans.

3. Substitution of alloxan in both imino groups, or in the C_5 position, abolishes its diabetogenic effect.

4. While the diabetogenic activity of the monosubstituted alloxans de-

creases with increasing length of the aliphatic side chain, the acute toxicity, caused chiefly by damage to the lung capillaries, increases. The introduction of an aromatic side chain results in a distinct fall in toxicity. Kidney lesions, as indicated by uremia, albuminuria, and anuria, are caused both by diabetogenic and non-diabetogenic compounds of this series.

5. A number of substances which give a strong Strecker reaction (deamination and decarboxylation of amino acids) are ineffective in inducing diabetes. 1,2-Naphthoquinone-4-sulfonic acid produces some disturbance of carbohydrate metabolism, but not true diabetes.

6. The diabetogenic potency of alloxan and some of its homologues, as expressed in terms of the effective dose 50, is not correlated with the Strecker reaction of these compounds (rate of murexide formation from amino acids).

7. Alloxan and some non-diabetogenic compounds cause a rapid fall in blood glutathione, but fail to affect the glutathione content of pancreas, liver, and kidney. This is taken as evidence against the view that alloxan diabetes depends on SH oxidation.

Mechanisms by which alloxan may damage the pancreatic islet cells are discussed in the light of these findings.

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THE INHIBITORY ACTION OF NAPHTHOQUINONES ON RESPIRATORY PROCESSES

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The first observation that certain 2-hydroxy-3-alkylnaphthoquinones exert an inhibitory action on respiratory processes was made by Wendel (19) during a study of the action of these compounds on malarial parasites. Wendel found that the relative effectiveness of these compounds in inhibiting the respiration of malarial parasites *in vitro* paralleled their ability to suppress malaria in animals. Our interest in these observations was aroused through our wartime collaboration with Dr. L. F. Fieser and his group, who were engaged in the synthesis and study of these naphthoquinones as potential antimalarial drugs. The discovery of Wendel suggested that these naphthoquinones might be general respiratory poisons and that a simple oxidative enzyme system might be found which was inhibited by them. If this were so, it was reasoned that such an enzyme system might be employed to determine the relative potency of new naphthoquinone derivatives, and thus serve as a relatively simple screening test for the prompt guidance of the organic chemist synthesizing these compounds.

We present data here to indicate that certain 2-hydroxy-3-alkylnaphthoquinones are very potent general respiratory poisons and that they act just below cytochrome *c* in the chain of respiratory enzymes. The succinate oxidase system is completely inhibited by certain of these compounds at concentrations as low as 3×10^{-6} M. The use of this enzyme system for testing the possible antimalarial activity of these drugs has been explored in Dr. Fieser's laboratories and will not be dealt with here.

EXPERIMENTAL

The various naphthoquinones employed were kindly supplied by Dr. L. F. Fieser or Dr. M. T. Leffler, or were those used by one of us in previous studies (4, 5). In all cases, a weighed amount of the compound was dissolved in absolute alcohol. Aliquots of the alcoholic solution were then evaporated to dryness on the water bath, and the residue dissolved in a drop or two of 0.1 N NaOH and made up to the desired concentration with isotonic NaCl solution or with water. All concentrations of the drugs reported here are in terms of the concentration in the final enzyme or cell test system.

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Unless otherwise stated, the various oxidative reactions were measured with the conventional Warburg manometric or Thunberg tube methods. When the manometric procedure was used, the amounts of cellular or enzyme material were so adjusted that the rate of oxygen consumption in the control usually fell in the range 80 to 150 c.mm. per hour. A solution of the naphthoquinone was added initially to the main chamber of the vessel, or was dumped from its side arm during the experiment.

Succinate oxidase was prepared from heart muscle by the following procedure:[1] Fresh beef heart was thoroughly minced and 50 gm. were well mixed with 200 cc. of ice-cold water. This suspension was then blended in a Waring blendor for 2 minutes at a temperature of 4–6° to form a heart homogenate. This homogenate was centrifuged for 10 minutes in the cold and the clear red supernatant discarded. This residue was next thoroughly mixed with 200 cc. of an ice-cold extract of heart muscle (prepared by heating to boiling a 50 gm. portion of minced heart muscle suspended in 200 cc. of water and filtering). The resulting suspension was then centrifuged in the cold and the clear, slightly red supernatant again discarded. If water or salt solutions are used in place of a heart muscle extract for washing at this stage, turbid supernatants are obtained with a consequent loss in enzyme. The insoluble residue was next washed with 200 cc. of cold water and centrifuged. The residue, which was now practically free of hemoglobin and myoglobin, was then blended in the Waring blendor for 30 seconds with a volume of ice-cold 0.1 M Na_2HPO_4 sufficient to give a total volume equal in amount to that of the original heart homogenate. This suspension upon the addition of cytochrome c showed on the average 95 per cent of the activity of the original heart homogenate. This final suspension was centrifuged for 10 minutes and the slightly turbid supernatant which contained approximately 50 per cent of the total enzyme was employed as the enzyme solution. A 6-fold concentration of the enzyme is effected by this procedure.

This preparation may be reduced to the dry state if it is rapidly frozen and desiccation carried out under vacuum from the ice phase. Some loss in activity is encountered by this procedure, but the dry powder so obtained remains active for weeks if it is kept dry and stored in the cold. Care must be exercised in reconstituting a suspension from the dry powder to form a thick smooth paste before diluting to volume.

The enzyme system was buffered with 0.1 M phosphate buffers. Cytochrome c, prepared according to the procedure of Keilin and Hartree (11), was added in sufficient quantity to insure saturation of the system with this component. Sodium succinate at a final concentration of 0.015 M was employed as substrate.

[1] Ball, E. G., and Ormsbee, R. A., unpublished data.

The succinate oxidase preparation was also used as a source of cytochrome oxidase and succinate dehydrogenase. Cytochrome oxidase activity was measured in the same way as succinate oxidase activity, except that p-phenylenediamine or hydroquinone was used as substrates. Succinate dehydrogenase activity was measured by the usual Thunberg tube technique, or manometrically, as will be described in presenting the experimental results.

Red blood cells, either parasitized or normal, were obtained from our own colony of monkeys (3, 9). We used 1 cc. of blood and 1.7 cc. of an isotonic phosphate-Locke's solution in each Warburg vessel (13). The phosphate-Locke's solution has a pH of 7.3 and the following composition: KH_2PO_4 0.011 M, Na_2HPO_4 0.044 M, NaCl 0.070 M, KCl 0.005 M, $CaCl_2$ 0.001 M.

The respiration of yeast cells was measured on a suspension of cells made from a Fleischmann yeast cake with glucose or lactate as substrate. The phosphate-Locke's solution described above was employed as suspending medium.

Liver slices from rabbit or monkey liver were also suspended in phosphate-Locke's solution. Glucose again served as the substrate.

Results

A comparison of the inhibitory action of six different hydroxynaphthoquinones on various respiratory systems is afforded by the data given in Table I. The six compounds listed there differ in structure only in the side chain substituted in the 3 positions of the naphthoquinone nucleus. Considering first the results obtained on succinate oxidase and the malarial parasite, *Plasmodium knowlesi*, it will be seen that the greatest inhibitory action is displayed by the two compounds containing a 9 carbon side chain, SN 5949 and SN 5090. Decrease in inhibitory activity results as the side chain is shortened. Hydrolapachol with a 5 and phthiocol with a 1 carbon side chain are much less effective compounds. Diminution of activity also results from the introduction of a double bond or of a hydroxy group into a side chain. This may be seen by comparing the action of hydrolapachol, lapachol, and hydroxyhydrolapachol. The action of these six naphthoquinones on *Plasmodium knowlesi* confirms the findings of Wendel (19), who has studied a much larger series of compounds. As stated earlier, Wendel also found that the antimalarial activity of a drug *in vivo* paralleled its ability to repress the respiration of the malarial parasite. Now, as can be seen from the data of Table I, there is a marked similarity in the action of these compounds on the succinate oxidase system and upon the respiration of the malarial parasite. This relationship suggests two things: first, the use of succinate oxidase as a test system for screening new drugs in this series, and second, the presence in the malarial parasite of an enzyme similar in nature to the one inhibited in the succinate oxidase preparation.

TABLE I

Comparison of Inhibitory Action of Hydroxynaphthoquinones on Various Respiratory Systems at pH 7.4 and 38°

The values* are expressed as per cent inhibition of O_2 consumption during the 1st hour of the experiment.

Hydroxynaphthoquinone	Succinate oxidase			Plasmodium knowlesi			Yeast			Liver slices	
	concentration										
	1	10	100	1	10	100	0.1	1.0	10	1.0	10
SN 5949† $R \cdot CH_2CH(CH_2)_5 \cdot CH_3$ (CH₃ branch)	94	100		63	75	74	58	95	94	0	16
SN 5090† $R \cdot CH_2CH_2CH_2CH$ (cyclohexyl)	69	85		27	75			0	15	0	0
Hydrolapachol $R \cdot CH_2CH_2CH(CH_3)_2$		0	88	10	37	78					
Lapachol $R \cdot CH_2CH{=}C(CH_3)_2$		26		0	0	74					
Hydroxyhydrolapachol $R \cdot CH_2CH_2C(CH_3)_2OH$		0			0	9					
Phthiocol, $R \cdot CH_3$		0	31			48					

* All the values are the average of two or more experiments.

† The Survey number, designated SN, identifies a drug in the records of the Survey of Antimalarial Drugs established by the Committee on Medical Research.

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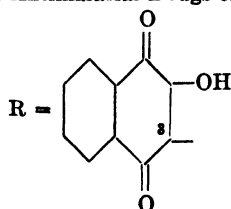
The side chain listed in the table is in all cases substituted in the 3 position.

In the results presented in Table I for yeast and liver slices, a marked difference in behavior is found. The respiration of liver slices during the

1st hour of the experiment is practically unaffected by the addition of either SN 5949 or SN 5090. Yeast respiration, on the other hand, though not affected appreciably by SN 5090, is as sensitive to SN 5949 as either the malarial parasite or succinate oxidase. These results might be interpreted as indicating a difference in respiratory mechanism for these cells. We are inclined to believe, however, that it represents a difference in permeability of the cells concerned for these compounds. The fact that yeast cells are inhibited by SN 5949 but not by SN 5090, which contains a cyclic group in the side chain, seems to us to favor this latter interpretation. Moreover, though the respiration of liver slices is practically unaffected by these compounds during the 1st hour of the experiment, an inhibiting effect begins to appear at the end of this time. A similar delayed action of SN 5949 on the respiration of heart muscle slices has been observed by Dr. O. H. Pearson in one experiment he kindly performed for us. In contrast to the delayed and weaker action on mammalian cells of these compounds is their practically instantaneous reaction on the parasitized red blood cell and succinate oxidase. Also, the full action of SN 5949 on the respiration of yeast cells becomes manifest within a few minutes after its addition. One cannot but wonder whether the delayed penetration of animal tissues by these drugs is not one important factor which helps to explain how such potent respiratory inhibitors can be used for therapeutic purposes. Certainly such tissue cells do contain a respiratory system sensitive to these compounds, as is shown by the results with the succinate oxidase preparation made from heart muscle.

It should also be pointed out that the maximum amount of inhibition obtainable varies according to the test system employed. For example, SN 5949 can cause complete inhibition of succinate oxidase; with yeast cells the inhibition has not exceeded 95 per cent, while, in the case of red blood cells parasitized with *Plasmodium knowlesi*, the inhibition has reached only 80 per cent Likewise, the respiration of the eggs of *Arbacia punctulata*, either fertilized or unfertilized, is markedly though incompletely blocked by SN 5949 (*cf.* Anfinsen and Ball (2)). This incomplete blockage of respiration in the intact cell is reminiscent of the action of cyanide. It suggests that these naphthoquinones, like cyanide, inhibit the main respiratory pathway, but leave certain secondary and minor respiratory processes untouched.

All the results so far described have been obtained at a pH of 7.4. The action of these naphthoquinones is, however, markedly dependent upon pH. Wendel (19) has already shown in the case of the malarial parasite that, over a range of pH which includes physiological values, unit decrease in pH produced approximately a 10-fold increase in antirespiratory activity for eight naphthoquinones that he studied. A similar effect of pH on the activity of SN 5949 and SN 5090 can be observed in the case of succinate

oxidase. In Fig. 1, data are presented which illustrate this effect. Here the concentration of naphthoquinone is held constant at 1 mg. per liter and the pH varied throughout the range 6.1 to 8.1. The enzyme is completely inhibited at the more acid values, but, as the pH becomes alkaline, the per cent inhibition falls as low as 20 per cent in the region of pH 8.0. Experiments performed with varying concentrations of SN 5949 in the pH range 6.1 to 6.6 indicate that pH changes in this region do not have the pronounced effect observed for the region pH 7.0 to 8.0. Studies made at pH values more alkaline than 8.1 with higher concentrations of SN 5949 indicate that its inhibitory activity continues to diminish somewhat in this region. However, results in this region are complicated by the fact that the activity of the succinate oxidase is diminished considerably as a result
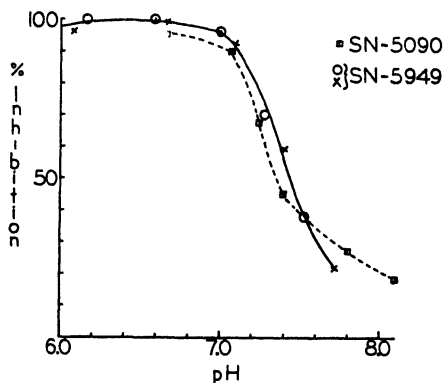
FIG. 1. The effect of pH on the inhibitory action of SN 5090 and SN 5949 on the succinate oxidase system. The concentration of naphthoquinone is 1 mg. per liter in all cases, temperature 37°; 0.1 M phosphate buffers were used throughout.

of the change in buffer composition and pH alone. It would, nevertheless, appear that the chief effect of pH occurs within the region shown in Fig. 1.

The reader will probably notice that in Fig. 1 a concentration of SN 5949 of 1 mg. per liter at pH 7.4 causes an inhibition of about 59 per cent, while, in Table I, an inhibition of 94 per cent is reported at this concentration and pH. This is due to the fact that different batches of succinate oxidase were employed in each case and serves to emphasize the point that such variations in sensitivity are encountered in different enzyme preparations. It should also be noted, in this connection, that fresh succinate oxidase preparations are always less sensitive than those which have been reconstituted from the dried preparation described in the experimental section.

The reason for variations of naphthoquinone action with the change in pH is not clear. One interpretation would be that it is due to alterations in the degree of dissociation of the hydroxy group of these compounds.

This would mean that the undissociated and least soluble form would be the most active. This explanation seems unlikely when one considers the fact that the pK of this hydroxy group in lapachol, hydroxyhydrolapachol, phthiocol, and four other hydroxynaphthoquinones not studied here has been found to be about 5.0 (Ball (5)). The pK value of SN 5949 and SN 5090 should be similar. Another explanation is that some essential group in the enzyme which reacts with the naphthoquinone undergoes ionization within this pH range. If this is the case, its pK value must be in the neighborhood of 7.5. A third possibility is that the effective concentration of the naphthoquinone is lowered at higher pH values, due to its increased affinity for proteins in general at more alkaline reactions. The succinate oxidase preparation is far from pure and undoubtedly contains many proteins not directly concerned in the enzyme reaction under study. In support of this explanation is the finding of Wendel (19) that serum proteins markedly affect the inhibitory action of these naphthoquinones on the respiration of malarial parasites. Which, if any, of these explanations is valid must await further experimentation.

We have attempted to learn the specific point of action of these naphthoquinones on general respiratory processes by a closer investigation of their action on the complex succinate oxidase system. The preparation employed as a source of this enzyme contains the following known components of the main respiratory pathway: cytochrome oxidase, cytochromes a, b, and c, and a flavoprotein capable of reoxidizing the reduced pyridine nucleotides. How many of these components are concerned in the oxidation of succinate is not known. However, cytochrome oxidase and cytochrome c are definitely known to be involved. It is possible to test the action of these two components separately from all the others by using p-phenylenediamine or hydroquinone as substrates instead of succinate. When this is done, it is found that no inhibition of the enzyme-catalyzed oxygen uptake by these substrates can be produced by SN 5949 or SN 5090 at final concentrations as high as 10 mg. per liter. Further evidence that cytochrome oxidase or cytochrome c is not affected by these naphthoquinones may be obtained spectroscopically. If a solution of cytochrome c, prepared by the method of Keilin and Hartree (11), is reduced with $Na_2S_2O_4$ at pH 7.4, the 550 mμ band of the reduced form may be observed with a hand spectroscope. This band disappears immediately upon addition of a little of the succinate oxidase preparation and shaking in the presence of air. Repetition of the test in the presence of SN 5949 at a final concentration of 10 mg. per liter gives identical results. In marked contrast are the results obtained in the presence of 0.001 M cyanide. In this case, the absorption band of reduced cytochrome c remains visible even after prolonged shaking of the solution with air. It is, therefore, obvious that these naphthoquinones do not in-

hibit respiration in the same manner as cyanide, namely, by reacting with cytochrome oxidase.

Another component of the succinate oxidase complex that may be studied is succinate dehydrogenase. The exact nature of this component is not known. Its catalytic action, however, may be followed independently of the functioning of cytochrome oxidase and cytochrome c by the use of methylene blue and the Thunberg tube technique. Employing this technique, it is found that the reduction time of methylene blue at pH 7.4 by succinate in the presence of the enzyme is slightly slowed by the presence of SN 5949 at a final concentration of 10 mg. per liter. That the positively charged methylene blue is not reacting with the anionic naphthoquinone and so interfering with its inhibitory action is shown by the fact that similar results are obtained when an indophenol type of oxidation reduction indicator is employed.

A more accurate picture of the action of SN 5949 on succinate dehydrogenase may be obtained by a manometric procedure. The results of an experiment of this type are shown in Fig. 2. In this test, cyanide has been added to all flasks, except Flask 1, in sufficient quantity to inhibit oxygen uptake by blocking the cytochrome oxidase system. In addition to cyanide, Flasks 3, 4, and 5 contain SN 5949 at a final concentration of 1, 5, and 10 mg. per liter, respectively. In Flask 1, it will be seen that oxygen consumption by succinate proceeds rapidly from the start of the experiment, since no inhibitor is present. In the other flasks, no oxygen consumption occurs until methylene blue is added from the side arm of the flask at 10 minutes after the start of the experiment. In these flasks, methylene blue acts as a by-pass to the cyanide-inhibited cytochrome oxidase. It will be noted, however, that the rate of oxygen uptake through this methylene blue by-pass is slower in those flasks, Nos. 3 to 5, which also contain SN 5949, but that complete blockage of the methylene blue-catalyzed system is not obtained. Though not shown in Fig. 2, a sixth flask containing 100 mg. per liter of SN 5949 gave exactly the same results as Flask 5, which contained 10 mg. per liter. This action of SN 5949 is in marked contrast to its behavior when cytochrome oxidase links the system to oxygen. With the enzyme preparation used for the experiment shown in Fig. 2, 1 mg. per liter of SN 5949 completely inhibits the oxygen uptake of the cytochrome oxidase-mediated system. From the results for Flasks 2 and 3 in Fig. 2, it may be calculated that 1 mg. per liter of SN 5949 causes only a 25 per cent inhibition of the methylene blue-linked system. These results would seem to indicate that the point of attack of the naphthoquinones is not upon the succinate dehydrogenase itself, but upon some enzyme or enzymes which link it to the cytochrome c-cytochrome oxidase chain, or, as in these experiments, to the methylene blue system. In the latter case, the inhibitory

effect is less marked than in the former, suggesting that a reaction involving the reduction of cytochrome c may be involved.

As may be surmised from the results in Fig. 2, methylene blue will partially release the inhibition of oxygen uptake caused by SN 5949. Results identical to those shown for Flasks 3, 4, and 5 in Fig. 2 are obtained when naphthoquinone is the only inhibitor present. Also the inhibition of yeast respiration by SN 5949 may be partially released by the addition of methylene blue. This is true whether glucose or lactate serves as substrate.
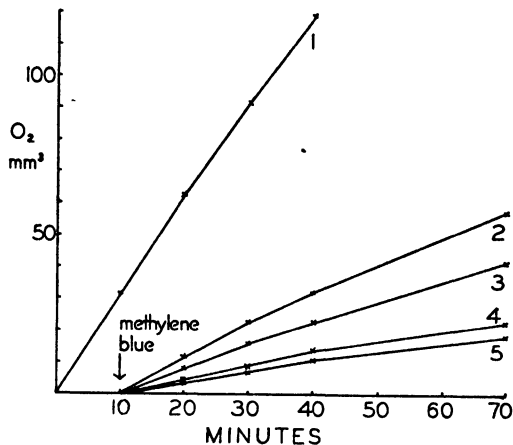
FIG 2   The effect of SN 5949 on the succinate dehydrogenase system, pH 7.4, and 37°. In addition to the succinate oxidase preparation, succinate and 0.1 M phosphate buffer, the various flasks contained the following: Flask 1, no additions; Flask 2, cyanide 0 001 M, Flask 3, cyanide 0 001 M, SN 5949, 1 mg. per liter; Flask 4, cyanide 0.001 M, SN 5949, 5 mg. per liter, Flask 5, cyanide 0.001 M, SN 5949, 10 mg. per liter. Methylene blue, 0.2 cc. of 0 1 per cent, dumped into Flasks 2 to 5, 10 minutes after the start of the experiment. The total volume in each flask is 3.0 cc. See the text for further details.

Further insight as to the locale of action of these naphthoquinones may be obtained by spectroscopic means. If a preparation of succinate oxidase, to which sodium succinate has been added, is viewed with a hand spectroscope, the absorption bands of reduced cytochromes a, b, and c are clearly visible. All these bands may be made to disappear momentarily or to fade in intensity if the preparation is shaken with air. They return promptly on standing. When the experiment is performed in the presence of SN 5949 at a final concentration of 20 mg. per liter, a different picture results. Shortly after mixing all the components, the only cytochrome band that is visible is that of reduced cytochrome b. Gradually, however, the reduced bands of cytochromes a and c appear. If this preparation is now shaken with air, the bands of reduced cytochromes a and c disappear immediately,

but those of reduced cytochrome *b* remain unaltered, even after prolonged shaking. On standing, the reduced bands of cytochromes *a* and *c* slowly reappear. Identical results may be obtained on a suspension of yeast cells to which glucose has been added as a substrate. These findings thus indicate that these naphthoquinones interfere with the reoxidation of reduced cytochrome *b*, but do not interfere with its reduction.

Reactions catalyzed by those enzymes which require the pyridine nucleotides as coenzymes do not appear to be affected by the naphthoquinones studied here. This fact was first indicated by the results of Wendel (19), who showed that the production of lactate from glucose by parasitized red blood cells was not inhibited, and indeed was enhanced, by the presence of some of these compounds. We have found no effect of SN 5949 on the mammalian lactate dehydrogenase system, as studied by the method of Quastel and Wheatley (14), nor upon the oxygen consumption of normal mammalian red blood cells that occurs in the presence of methylene blue and glucose. Both of these processes require the presence of pyridine nucleotides. We have also been unable to observe any effect by them upon the reoxidation of reduced diphosphopyridine nucleotide by methylene blue, as catalyzed by the flavoprotein described by Corran *et al.* (7). The main effect of these naphthoquinones on respiration thus appears to be limited to an action upon some unknown component gearing the dehydrogenases to the cytochrome oxidase-cytochrome *c* system.

Xanthine oxidase, *d*-amino acid oxidase, catalase, and urease are unaffected by SN 5949. The latter enzyme was tested mainly because its activity, like that of succinate oxidase, is dependent upon the presence of sulfhydryl groups. The findings of Colwell and McCall (6) that the antibacterial action of 2-methyl-1,4-naphthoquinone is antagonized by various sulfhydryl compounds also suggested that SN 5949 might be reacting with an enzyme sulfhydryl group. We have attempted without success to protect the succinate oxidase system against the action of SN 5949 by the addition of cysteine.

DISCUSSION

It is not possible at the present time to ascertain the exact manner in which naphthoquinones of the type used here block respiratory processes. We may list the various enzymes that may be involved in the main respiratory pathway in the manner portrayed in Table II. They are listed here in the order of their relative positions in this pathway in so far as this can be judged from available data. The exact rôle of several enzymes listed remains in doubt. For example, the function of cytochrome *a* is unknown and its possible relationship or even identity with cytochrome oxidase is still an open question. The same is true for cytochrome *b* and the possibility

exists that it and succinate dehydrogenase may be identical. Another disputable point is whether succinate dehydrogenase, whatever its identity, forms a part of the main respiratory pathway. Until separation of all the individual components concerned in this chain is achieved, these questions will be difficult to answer. Nevertheless, it is possible to ascertain the level at which certain substrates are geared into this chain of oxidative enzymes. In the column headed "Substrate" in Table II, the substrates p-phenylenediamine, succinate, and glucose have been listed in a position opposite that enzyme in the chain which activates its electrons for passage through the chain. Reduction of all components of the chain lying above this specific enzyme may be assumed to occur in the presence of the specific substrate and can be shown to occur in most instances.

TABLE II

Relationships of Enzymes, Substrates, and Inhibitors in Main Oxidative Pathway

Inhibitor	Enzyme system	Substrate
Cyanide	Cytochrome oxidase	
	" a	
	" c	p-Phenylenediamine, etc.
Naphthoquinone	?	
	Cytochrome b	
	Succinate dehydrogenase	Succinate
	Flavoprotein (diaphorase)	
	Pyridine nucleotide dehydrogenases	Glucose, etc.

Now the relative position at which cyanide and 2-hydroxy-3-alkylnaphthoquinone inhibit this chain may be similarly indicated. In the column headed "Inhibitor" cyanide is placed opposite cytochrome oxidase, since it is this lead off enzyme in the chain with which it appears to combine. The oxidation of all the substrates listed is prevented by its action. In the case of the naphthoquinones, their point of action is indicated as lying between cytochromes b and c, since they do not prevent the oxidation of p-phenylenediamine; nor do they prevent the reduction of cytochrome b by either succinate or glucose. Neither cytochrome c nor cytochrome b can, therefore, be singled out as the enzyme reacting with the naphthoquinones. It is the interaction of these two enzymes which appears to be prevented by the naphthoquinones. This being the case, the naphthoquinones might be classified as inhibitors preventing the interaction of two specific enzymes rather than completely inhibiting the action of one, as usually happens. Though this would be an unusual situation, it must, nevertheless, be remembered that these reactions are unusual in that we are dealing with the inter-

action of two enzymes and not, as in the more conventional manner, with enzyme and substrate.

An alternative hypothesis must, however, be considered. This is that an unknown enzyme exists which mediates the reaction between cytochromes b and c in the chain and that it is this unknown enzyme which specifically reacts with naphthoquinones. Certainly we have no proof that cytochrome b can react directly with cytochrome c. Indeed, evidence (10, 12, 16) has been accumulating that some unknown enzyme acting below cytochrome c is involved in the oxidation of succinate. Most recently Straub (17) has presented evidence for the existence of a so called SC factor linking succinate dehydrogenase and cytochrome c. He pictures the oxidation of succinate as proceeding through the following chain: succinate dehydrogenase $\rightarrow$ SC factor $\rightarrow$ cytochrome c $\rightarrow$ cytochrome a $\rightarrow$ cytochrome oxidase. The possible relationship of cytochrome b to this factor or to succinate oxidation is not discussed by him. However, the term "succinate dehydrogenase" is used by Straub, as we have used it, to describe the unidentified enzyme or enzymes catalyzing the reaction between methylene blue and succinate. As mentioned earlier, cytochrome b may well be involved in this reaction. Indeed, the fact that cytochrome b is present in the succinate dehydrogenase preparation and is rapidly reduced upon the addition of succinate favors such an interpretation. There is thus the possibility that Straub's SC factor actually links cytochrome b and cytochrome c and that it is indeed the enzyme which reacts with naphthoquinone.

This interpretation of the action of naphthoquinones is satisfactory in the light of Straub's findings only so far as the oxidation of succinate is concerned. When we attempt to explain the blockage of general respiration in terms of the action of naphthoquinones on Straub's SC factor, the picture is not clear. For example, in the oxidation of glucose we may trace the pathway of electrons from this substrate through the pyridine nucleotides and the flavoprotein, diaphorase. Beyond this we cannot go. How this flavoprotein is linked to oxygen is not known, but the cytochrome system is undoubtedly involved. Straub (18) has shown that direct reaction between diaphorase and cytochrome c does not occur. Some enzyme or enzymes must then lie intermediate to these two components. Now Straub (17) has attempted without success to gear diaphorase to cytochrome c by means of his SC factor. Indeed, in his experiments the same combination of enzymes capable of functioning as succinate oxidase failed to connect diaphorase to cytochrome c. Whether the SC factor is involved in general respiration cannot then be determined from Straub's experiments, since the possibility exists that the SC factor in combination with some component missing in his experiments is involved in linking diaphorase to cytochrome c.

From this discussion then it is obvious that there is a portion of the main

oxidative chain lying below cytochrome c which is shrouded in darkness. It is somewhere within this region that the naphthoquinones act to block not only succinate oxidation but also general respiration. Perhaps these compounds may prove to be useful tools to help unravel the nature of the enzyme or enzymes which gear cytochrome c not only to succinate dehydrogenase but also to the flavoprotein-pyridine nucleotide systems.

One other point of interest that should be mentioned with regard to the 2-hydroxy-3-alkylnaphthoquinones is that they are natural constituents of certain living cells. Lapachol, one of the compounds listed in Table I, occurs in a variety of woods; *cf.* Fieser (8). Lomatiol, a closely related compound, occurs in the seeds of *Lomatia ilicifolia* (15). Phthiocol, also listed in Table I, is a pigment isolated from the tubercle bacillus by Anderson and Newman (1). Using the data given by these authors, we estimate roughly that the concentration of phthiocol in this organism is of the order of 10 mg. per kilo of wet cells. This is just below the concentrations found necessary to inhibit succinate oxidase or the respiration of the malarial parasite. Many other naphthoquinones, though not of this general type, are also of course found in nature. Among those may be mentioned vitamin K, juglone, lawsone, lomatiol, and echinochrome. With the exception of vitamin K, no function is apparently known for any of these compounds. We have considered the possibility that the inhibitory action of compounds such as SN 5949 may be due to their competition with a naturally occurring naphthoquinone. Preliminary experiments in which an attempt was made to release respiratory inhibition produced by SN 5949 by the addition of vitamin K were inconclusive. Our observations (2) that the respiration of the *Arbacia* egg, which contains large amounts of echinochrome, is easily inhibited by SN 5949 do not favor a competitive type of action. Nevertheless, the findings reported here suggest that an investigation of the rôle of naturally occurring naphthoquinones either in regulating or participating in respiratory processes might be of interest.[2]

SUMMARY

1. Certain 2-hydroxy-3-alkylnaphthoquinones are very potent inhibitors of respiratory processes. Their activity depends upon the nature of the alkyl group.

2. The more effective naphthoquinones completely inhibit the oxygen uptake of the succinate oxidase system at concentrations as low as 3×10^{-6}

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[2] Since submitting this paper for publication the interesting work of Gaffron (20) has come to our attention. He reports that phthiocol first stimulates and then inhibits the respiration of certain algae as its concentration is increased. He also shows that phthiocol interferes with the photochemical processes of these plant cells and discusses his findings in relation to the known high vitamin K content of chloroplasts.

M. The respiration of the malarial parasite, *Plasmodium knowlesi*, and of yeast cells is also inhibited at similar concentrations, but inhibition never exceeds 80 to 95 per cent of the total respiration in these cases.

3. The action of these naphthoquinones on the succinate oxidase system is dependent on the pH, at least within the region 7.0 to 8.0. The activity of a compound at pH 7.0 is approximately 5-fold that at pH 8.0. The reason for this effect is not known, but several possible explanations are offered.

4. Cytochrome oxidase, xanthine oxidase, *d*-amino acid oxidase, catalase, several pyridine nucleotide systems, and urease are unaffected by these naphthoquinones. The succinate dehydrogenase system is partly inhibited.

5. The conclusion is reached from manometric and spectrophotometric observations that these naphthoquinones act below cytochrome *c* and above cytochrome *b* in the main chain of respiratory enzymes The possibility that their inhibitory action is the result of their combination with an unknown enzyme which mediates the reaction between cytochrome *c* and cytochrome *b* is discussed.

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AMINO ACIDS EXCRETED BY MICE FED INCOMPLETE PROTEINS*

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In a previous study it was noted that mice fed a diet containing 10 per cent of casein excreted from 1 to 5 per cent of the ingested amino acids in the urine in microbiologically available form (1). The present experiments deal with the amino acids in the urine of mice fed incomplete proteins such as oxidized casein (2) supplemented in various ways. Oxidized casein is known to be deficient in methionine, tryptophane, and cystine (2, 3). Arachin was fed as an example of a natural protein low in one of the essential amino acids, while fibrin and casein served as examples of relatively complete proteins.

Methods

Weanling mice weighing 10 to 12 gm. were fed diets of the following general composition.

	per cent
Protein	10
Corn oil	5
Wesson's salt mixture (4)	4
Glucose monohydrate (cerelose) to	100

	γ per gm.
Pyridoxine hydrochloride	10
Thiamine chloride	6
Nicotinic acid	10
Calcium pentothenate	20
Riboflavin	6
Folic acid	0.5
Biotin	0.5
p-Aminobenzoic acid	300
Inositol	500
Choline chloride	1000
Cystine. ..	1000

Methionine was added to certain of the diets at a level of 0.7 per cent, tryptophane to certain others at a level of 0.4 per cent (Tables II to IV).

When the dietary protein was ordinary casein or fibrin, the mice gained an average of 2.5 gm. per week. Mice fed oxidized casein supplemented with methionine, tryptophane, and cystine also grew well and when the protein was finely ground, some series gained as much as 3.0 gm. per week. However, when methionine or tryptophane was omitted from the diet the animals lost weight at rates ranging from 0.5 to 2.0 gm. per week and some died after 2 or more weeks. For this reason the feeding periods were necessarily of short duration.

After 15 or more days on the various diets, mice were placed in metabolism cages in groups of three, and the urine was collected under toluene for periods of 24 or 48 hours. The urine was stored, diluted, and analyzed microbiologically for sixteen amino acids by the methods described previously (1).

The results were expressed both as the amounts of the various amino acids excreted in microbiologically available form per mouse daily and as the percentages of the ingested acids appearing in the urine. The latter values were originally calculated from published values for the amino acid composition of the proteins fed, but irregularities in the apparent percentages of some of the amino acids excreted, *e.g.*, of serine on the fibrin diet and of proline on arachin, suggested that the published values (5, 6) used might not reflect the composition of the proteins fed in the present experiments. Accordingly, each of the proteins (Table I) was hydrolyzed by autoclaving 600 mg. aliquots in sealed ampuls with 10 per cent HCl (5 N NaOH for tryptophane and tyrosine determinations) for 10 hours at 15 pounds pressure (7, 8). The pH was then adjusted to 6.8, the solution diluted, filtered, and the amino acid composition determined microbiologically[1] (1). With comparatively few exceptions, the percentages of the various amino acids in the proteins were found to be of the same order of magnitude as those reported by others (Table I). In line with the biological and chemical results of Toennies and Bennett (2, 3), most of the amino acids in casein were unaltered by the oxidation process, although methionine, cystine, and tryptophane were destroyed completely.

Results

The most obvious finding was that mice on a protein inadequate in the essential amino acids tryptophane (Table II) or methionine (Table III)

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[1] When *Lactobacillus arabinosus* was used as the test organism for the determination of glutamic acid, the value obtained for the percentage of this acid in casein compared favorably with those reported by others who used either chemical or microbiological methods of analysis (9–11). However, with *Streptococcus faecalis* and the regular test medium (1), percentage values for glutamic acid often differed from those obtained with *L. arabinosus*, unless the content of asparagine in the medium was doubled. In the present study, *L. arabinosus* was used routinely for the determination of glutamic acid in urine and in protein hydrolysates.

excreted abnormally high amounts of all the other amino acids. On the tryptophane-deficient diet (Series I) (oxidized casein plus methionine and cystine) the percentage of ingested amino acids appearing in the urine after 15 days varied from 12.8 per cent for proline to 48.3 per cent for histidine (Table II), while all other amino acids were found in the urine in intermediate percentages of the amounts ingested. Essentially the same values were obtained in Series II and III in which the period of depletion was more prolonged. The excretion of the essential amino acids taken

TABLE I

Per Cent Amino Acids in Proteins Fed (Gm of Amino Acid Liberated per 100 Gm of Air-Dried Protein)

Amino acid	Fibrin	Arachin	Ordinary casein	Oxidized casein	Recoveries*		
					Ordinary casein	Fibrin	Arachin
Arginine	6 8	11 0	3 7	5 8	94	103	101
Aspartic acid	15 5	14 0	7 3	8 3	100	107	104
Cystine	1 8	0 9	0 45	0 0	103	100	104
Glutamic acid	13 0	21 6	21 5	22 0	109	100	109
Histidine	2 5	2.6	3 0	2 4	94	100	109
Isoleucine	7 6	6 6	8 6	7 8	100	101	104
Leucine	6 7	6 9	10 5	10 4	107	105	107
Lysine	6 8	1 8	6 7	7 5	91	94	96
Methionine	2 6	1 1	3 1	0 0	103	92	100
Phenylalanine	3 8	5 5	4 8	5 0	93	104	102
Proline	4 6	6 0	15 9	16 9	101	104	101
Serine	11 2	9 9	7 9	7 5	101	101	100
Threonine	5 9	2 6	4 6	3 6	96	94	97
Tryptophane	3 6	0 69	1 4	0 0	105	103	103
Tyrosine	4 6	4 5	5 2	4 5		97	99
Valine	3 7	3 9	5 8	6 1	105	99	101

* Amino acids were added prior to hydrolysis in quantities approximately equal to those contained in the protein sample being hydrolyzed

collectively did not appear to differ from that of the non-essential amino acids.

Similar results were obtained when the deficient amino acid was methionine (Table III). After 15 days of depletion, mice fed 10 per cent of oxidized casein plus tryptophane and cystine (Series I) excreted 9.9 per cent of the ingested aspartic acid in the urine and 37.5 per cent of the ingested histidine; all other amino acids were excreted in intermediate percentages. In Series II the percentages of the different amino acids excreted ranged from 24.6 for glutamic acid to 86.5 per cent for histidine. Both in this and in other series the percentage excretion of amino acids fed in the free form (cystine and methionine or tryptophane) appeared

to be higher than those for most of the amino acids fed combined as protein, but no particular significance was attached to this observation since the possibility existed that some free amino acid added to the diet may have been spilled in the metabolism cage and thus have contributed to the high urinary values observed.

TABLE II

Amino Acids in Urine of Mice Fed Diet Low in Tryptophane (10 Per Cent Oxidized Casein Plus Methionine and Cystine)

Amino acid	Series I*			Series II†	Series III‡
	Amino acid ingested	Amino acid excreted	Ingested amino acid excreted	Ingested amino acid excreted	Ingested amino acid excreted
	mg	*mg.*	*per cent*	*per cent*	*per cent*
Arginine	11 02	1 70	15.4	14.5	17 9
Aspartic acid	15 77	2 14	13.6	20 3	19 4
Cystine	1 90	(0 70)	(36 8)	(196 0)	(67 0)
Glutamic acid	41 80	5 97	14 3	19 3	17 8
Histidine	4 56	2 20	48 3	46 4	71 0
Isoleucine	14 82	2 92	19 7	24 0	25 7
Leucine	19 76	5 28	26 7	27 9	30 3
Lysine	14 25	3 36	23.6	27 2	23 5
Methionine	5 70	(1 72)	(30.2)	(50 0)	(43 5)
Phenylalanine	9 50	2 33	24 6	25 6	30 0
Proline	32 11	4 10	12 8	15 5	19 1
Serine	14 25	2 82	19 9	16 8	23 6
Threonine	6 84	2 90	42 5	45 5	40 7
Tryptophane	0	0 15			
Tyrosine	8 55	1 80	21 0	22 2	24.9
Valine	11 59	3 22	27 8	23 4	27 0

The numbers in parentheses are not directly comparable to the other values, since it is possible that some of the free amino acids fed may have been spilled into the urine.

* Series I was fed the low tryptophane diet for 15 days before the urine was collected.

† Series II was fed the low tryptophane diet for 10 days, then fed the same diet plus tryptophane for 12 days, followed by the low tryptophane diet for 14 days before the urine collection was made.

‡ Series III was fed the low tryptophane diet for 18 days before the urine collection was made.

When oxidized casein was supplemented with methionine, tryptophane, and cystine, the excretion of all amino acids except those added in the free form was reduced to 10 per cent of the amount ingested or less (Table IV) and usually was about 5 per cent. In fact, the excretion of microbiologically available amino acids was essentially the same on the properly

supplemented oxidized casein as when ordinary untreated casein was fed at a comparable percentage in the diet.   This similarity suggested that the rise in the excretion of amino acids noted on the deficient diets containing oxidized casein was due to the deficiency in an essential amino acid rather than to any inherent property of the oxidized casein.   This was

TABLE III

Amino Acids in Urine of Mice Fed Diet Low in Methionine (10 Per Cent Oxidized Casein Plus Tryptophane and Cystine)

Amino acid	Series I*			Series II†
	Amino acid ingested	Amino acid excreted	Ingested amino acid excreted	Ingested amino acid excreted
	mg	*mg*	*per cent*	*per cent*
Arginine	9 86	1 08	11 0	31.1
Aspartic acid	14 11	1 40	9 9	26.6
Cystine	1 70	(0 58)	(34 1)	(65 4)
Glutamic acid	37 40	5 17	13 8	24.6
Histidine	4 08	1 53	37 5	86 5
Isoleucine	13 26	1 80	13 6	37.3
Leucine	17 68	3 25	18 4	42.4
Lysine	12 75	1 58	12 4	34.3
Methionine		0 25		‡
Phenylalanine	8 50	1 50	17 7	42 5
Proline	28 73	3 60	12 5	25.9
Serine	12 75	1 58	12 4	31.4
Threonine	6 12	1 90	31 1	65.4
Tryptophane	2 04	(0 80)	(39 2)	(60.5)
Tyrosine	7 65	0 40	15 7	36 8
Valine	10 37	1 67	16 1	37 4

The numbers in parentheses are not directly comparable to the other values, since it is possible that some of the free amino acids fed may have been spilled into the urine

* Series I was fed the low methionine diet for 15 days before the urine was collected

† Series II was fed the low methionine diet for 18 days before the urine was collected

‡ 0.25 mg of methionine was found in the urine upon analysis, even though none was fed in the diet

further emphasized by the relatively high percentages of amino acids excreted in the urine when 10 per cent of arachin was fed (Table V).   Arachin contains only 1.1 per cent of methionine and when the arachin in the diet was 10 per cent, an average of only 3.5 mg. of methionine was ingested per mouse daily compared to 8.77 mg. in diets containing 10 per cent of casein.   The arachin diet therefore represented an inadequacy of an essen-

tial amino acid rather than a complete deficiency.   When the arachin diet
was fed for only 15 days, most of the amino acids were excreted in relatively
low amounts, but as the feeding period was prolonged, the excretion of
amino acids increased until by 63 days the percentages of ingested acids
appearing in the urine ranged from 8.8 for aspartic acid to 20.5 for lysine.

TABLE IV

*Amino Acids in Urine of Mice Fed Ordinary Casein or Oxidized Casein Properly
Supplemented (10 Per Cent Oxidized Casein Plus Methionine,
Tryptophane, and Cystine)*

Amino acid	Supplemented oxidized casein Series I*			Ordinary casein Series I†		
	Amino acid ingested	Amino acid excreted	Ingested amino acid excreted	Amino acid ingested	Amino acid excreted	Ingested amino acid excreted
	mg	*mg*	*per cent*	*mg*	*mg*	*per cent*
Arginine	11 60	0 30	2 6	11 47	0 67	5 8
Aspartic acid	16 60	0 83	5 0	22 63	0 95	4 2
Cystine	2 00	(0 44)	(22 0)	4 10	(0 26)	(6 4)
Glutamic acid	49 06	1 58	3 2	66 65	2 71	4 1
Histidine	4 80	0 48	10 0	9 30	0 83	8 9
Isoleucine	15 60	0 67	4 3	26 66	1 34	5 0
Leucine	20 80	1 01	4 9	29 72	1 01	3 4
Lysine	15 00	0 47	3 1	20 77	1 25	6 0
Methionine	6 30	(0 53)	(8 4)	8 77	0 28	3 2
Phenylalanine	10 00	0 53	5 3	14 88	0 86	5 8
Proline	33 80	0 94	2 8	49 29	1 82	3 7
Serine	15 00	0 63	4 2	24 49	1 46	6 0
Threonine	7 20	0 50	7 0	14 26	1 00	7 1
Tryptophane	2 52	(0 33)	(13 2)	4 34	0 20	4 6
Tyrosine	9 00	0 44	4 9	16 12	0 92	5 7
Valine	12 20	0 50	4 1	17 98	0 91	5 1

The numbers in parentheses are not directly comparable to the other values, since
it is possible that some of the free amino acids fed may have been spilled into the
urine

* Series I, fed the supplemented oxidized casein diet for 15 days before the urine
collection was made

† Series I, fed the ordinary casein diet for 44 days before the urine collection
was made

When fibrin was fed as 20 per cent of the diet, the percentage excretion of
all amino acids into the urine was low (Table VI).

Bound Amino Acids in Urine—Previous experiments (1) on urine from
mice fed different levels of casein indicated that at least half of the amino
acids in the urine existed in a combined form.   In the present study,
representative samples of urine from mice fed the various diets were hydro-

lyzed with 10 per cent HCl for 5 hours at 15 pounds pressure, and determinations were made of the α-NH$_2$-N by the copper method (12, 13), while histidine, threonine, arginine, and valine were determined microbiologically by the methods previously employed on unhydrolyzed urine. In line with previous results, roughly one-half of the amino acids existed in a bound form in the specimens of urine from mice fed complete proteins

TABLE V

Amino Acids in Urine of Mice Fed 10 Per Cent Arachin Diet

Amino acid	Series Ia*		Ingested amino acid excreted	Series Ib†	Series Ic‡
	Amino acid ingested	Amino acid excreted		Ingested amino acid excreted	Ingested amino acid excreted
	mg	*mg*	*per cent*	*per cent*	*per cent*
Arginine	35 20	1˙01	2 9	4 0	11 8
Aspartic acid	44 80	0 94	2 1	2 2	8 8
Cystine	6 08	(0 71)	(11 7)	(42 0)	(33 4)
Glutamic acid	69 12	1 97	2 6	2 7	10 0
Histidine	8 32	0 48	5 8	5 0	18 6
Isoleucine	21 12	0 40	1 9	2 5	12 2
Leucine	22 08	0 73	3 3	4 2	10 1
Lysine	5 76	0 16	2 8	3 5	20 5
Methionine	3 52	0 13	3 7	5 0	10 0
Phenylalanine	17 60	0 54	3 1	3 3	
Proline	19 20	0 60	3 1	2 6	13 1
Serine	31 68	0 61	1 9	2 9	
Threonine	8 32	0 20	2 4	3 4	13 5
Tryptophane	3 80	0 04	1 0	1 6	
Tyrosine	14 40	0 59	4 1	3 5	18 9
Valine	12 48	0 32	2 5	3 5	15 7

The numbers in parentheses are not directly comparable to the other values, since it is possible that some of the free amino acids fed may have been spilled into the urine

* Series Ia was fed the arachin diet for 15 days before the urine collection was made

† Series Ib was fed the arachin diet for 18 days before the urine collection was made

‡ Series Ic was fed the arachin diet for 63 days before the urine collection was made

such as fibrin, casein, or oxidized casein properly supplemented (Table VII). Urine from mice fed incomplete proteins contained approximately the same amounts of bound amino acids as normal urine, but since the abnormal urines contained greatly increased amounts of "free" amino acids, the percentages of the total existing in the bound form were relatively low, 11 to 16 per cent for the sample excreted on deficient diets compared to 48 to 57 per cent excreted on complete proteins (Table VII).

Microbiological determinations of four representative amino acids in the urine before and after hydrolysis tended to confirm the general conclusions from determinations of α-NH$_2$-N (Table VIII). Although there were fairly wide variations noted in the percentages of the four amino acids excreted in the bound form on the various diets, the total amounts excreted were usually lowest on the fibrin diet. Neither arginine nor valine was present in the bound form in the urine excreted on diets deficient in methio-

TABLE VI

Amino Acids in Urine of Mice Fed 20 Per Cent Fibrin Diet

Amino acid	Series Ia*			Series Ib†
	Amino acid ingested	Amino acid excreted	Ingested amino acid excreted	Ingested amino acid excreted
	mg	*mg*	*per cent*	*per cent*
Arginine	33 59	0 27	0 8	1 3
Aspartic acid	76 57	1 01	1 4	1 0
Cystine	11 36	(0 65)	(5 7)	(5 9)
Glutamic acid	64 22	1 22	1 9	3 7
Histidine	12 35	0 67	5 4	7 2
Isoleucine	37 54	0 55	1 5	1 1
Leucine	33 10	0 55	1 7	2 2
Lysine	33 59	0 75	2 2	1 5
Methionine	12 84	0 23	1 8	1 4
Phenylalanine	18 77	0 40	2 1	2 3
Proline	22 72	0 65	2 9	2 9
Serine	55 33	0 63	1 1	2 2
Threonine	29 15	0 70	2 4	
Tryptophane	17 78	0 30	1 7	0 5
Tyrosine	22 72	0 27	1 2	1 4
Valine	18 28	0 33	1 8	1 8

The numbers in parentheses are not directly comparable to the other values, since it is possible that some of the free amino acids fed may have been spilled into the urine.

* Series Ia was fed the fibrin diet for 44 days before the urine was collected.

† Series Ib was fed the fibrin diet for 16 days before the urine was collected

nine; furthermore, bound arginine was absent when tryptophane was missing from the diet. Bound arginine was present in the urine on the diets containing ordinary casein and the amounts were particularly high when fibrin was fed (Table VIII). This might suggest that arginine normally appears in mouse urine combined with methionine or tryptophane. However, a much more thorough study of the responses of microorganisms to various peptides will have to be made before valid conclusions can be drawn on the nature of the peptides in urine.

TABLE VII

Excretion of "Free" and "Bound" α-NH$_2$-N in Urine by Mice Fed Various Proteins

Diet	N ingested	NH$_2$-N excreted in urine daily		Bound NH$_2$-N in urine	NH$_2$-N excreted per mg. N ingested		Bound NH$_2$-N in urine per mg. N ingested	Per cent of total in "bound" form
		Un-hydro-lyzed	Hydro-lyzed		Un-hydro-lyzed	Hydro-lyzed		
	mg.	mg.	mg.	mg.	mg.	mg.	mg.	
Oxidized casein, no methionine	20 3	5 77	6 50	0.73	0.250	0 282	0 032	11
Oxidized casein, no tryptophane	30 4	4.92	5 78	0 86	0.162	0.190	0.028	16
Oxidized casein; supplemented	32 0	1.13	2.06	0.93	0.035	0.064	0.029	48
Ordinary casein	45 3	1 30	2.96	1.66	0.029	0 065	0.036	57
Fibrin	80 0	0 58	1.15	0 57	0.007	0.014	0.007	52
Arachin	32.0	4 82	5.53	0.71	0.150	0.173	0.023	13

TABLE VIII

Urinary Excretion of "Free" and "Bound" Amino Acids by Mice Fed Various Diets

Diet	Histidine excreted daily			Per cent of total in "bound" form	Threonine excreted daily			Per cent of total in "bound" form
	Un-hydro-lyzed	Hydro-lyzed	"Bound" form		Un-hydro-lyzed	Hydro-lyzed	"Bound" form	
	mg.	mg.	mg.		mg.	mg.	mg.	
Oxidized casein, no methionine	1.3	2.1	0 8	38	4.3	5 0	0.7	14
Oxidized casein, no tryptophane	1.4	1 8	0.4	22	3.9	4 3	0.4	9
Oxidized casein; supplemented	0 33	0 50	0 17	34	0.82	1.6	0.78	49
Ordinary casein	0 23	0.70	0.47	67	0 90	2.1	1.2	57
Fibrin	0 15	0 33	0.18	55	0 45	0.72	0.27	38
Arachin	1 0	1.3	0.3	23	1 9	3.2	1.3	41
	Arginine				Valine			
Oxidized casein, no methionine	3.9	3.7	0	0	2.1	2.1	0	0
Oxidized casein, no tryptophane	3.2	3 2	0	0	2.3	3.0	0.7	23
Oxidized casein; supplemented	0.50	0 60	0.10	17	0.30	0.73	0.43	59
Ordinary casein	0 66	1 2	0.54	45	0.70	1.7	1.0	59
Fibrin	0.28	1 6	1.32	83	0.64	0.75	0.11	15
Arachin	2.3	2.4	0.1	4	2.4	6.0	3.6	60

DISCUSSION

Normally, amino acids in the blood can be prevented from appearing in the urine by one or more of the following processes: (a) by the incorporation of the amino acids into the tissues for the synthesis of proteins and other substances; (b) by the destruction of amino acids through deamination, transamination, or other reactions; (c) by the kidney barrier. If any one of these processes were completely effective, no amino acids would appear in the urine.

Process (a) is limited even under optimal conditions of nutrition, for the tissues do not accumulate protein indefinitely. When an essential amino acid is missing from the diet, the synthesis of protein, even for replacement purposes, is curtailed severely for lack of an essential building block, and instead of passing from the blood stream into the tissues, amino acids pass from the tissues into the blood stream. Process (b) appears to be limited in the mouse, for when the percentage of casein in the diet is raised from 10 per cent to 50 per cent, the amounts of the various amino acids excreted in the urine also increase 5-fold (1). This latter observation further indicates that the kidney is not a particularly effective barrier against the transfer of amino acids from the blood of the mouse to the urine; in other words, process (c) is only partially effective.

In the absence of an essential amino acid, there is not only a failure of protein synthesis, but there may also be an impairment in the mechanisms by which amino acids are destroyed in the body. The amounts destroyed can be estimated from representative data as follows: Mice on the diet free from methionine ate 1.27 gm. of food daily, equivalent to 127 mg. of protein. They lost weight at a rate of 0.20 gm. per day, equivalent to 40 mg. of protein, if it is assumed that one-fifth of the lost weight is protein. (Qualitatively the results will be similar whether the increments of weight lost or gained are assumed to be 10 or 30 per cent of protein.) The amounts of amino acids entering the blood stream daily are therefore 127 + 40 mg. = 167 mg. 57.2 mg. were excreted in the urine as free acids or peptides, leaving 110 mg. destroyed daily. Similar calculations, applied to data from animals deficient in tryptophane, indicate a destruction of 179 mg. of amino acids daily.

When both tryptophane and methionine were fed in addition to 10 per cent of oxidized casein, a typical daily intake was 210 mg. of protein, with a gain in weight of 0.1 gm. daily, equivalent to an estimated absorption of 20 mg. of amino acids daily by the tissues. Since 20 mg. of amino acids were excreted daily, the amounts destroyed were 210 − (20 + 20) = 170 mg.

Thus, the amounts of the amino acids destroyed daily appear to be essentially of the same order of magnitude whether a "complete" diet is

fed on which there is a gain in weight, or an incomplete diet on which weight is lost. Nevertheless, the amounts of amino acids excreted on the incomplete diet are at least double those on the complete diet, 40 to 55 mg. per day *versus* 20 mg. By way of contrast, it can be calculated that normal mice fed a diet containing 50 per cent of casein destroy nearly 1500 mg. of amino acids daily. Thus the extra amino acids excreted on the deficient diets represent acids that would have been destroyed had the diet been complete. The implication is that the enzyme systems that normally destroy amino acids are impaired on the deficient dietary regimens. Quite another possibility is that the reabsorption of amino acids in the kidney is impaired on the incomplete diets.

Presumably, deficiencies in other essential amino acids would likewise result in a general increase in the excretion of amino acids by the mouse. The possibility also suggests itself that deficiencies in vitamins might affect amino acid excretion. Very little information has been published on this point, although it appears to be well established that pathological conditions involving liver damage can be accompanied by increases in the α-NH_2-N content of the urine (14). Kaplanskii *et al.* (15) noted that patients having injuries of the peripheral nervous system accompanied by severe pain excreted increased amounts of keto acids and amino acids in the urine, and that the administration of large doses of vitamin B_1 returned the excretion to a normal level. In a previous study in this laboratory, mice deprived of vitamin B_6 excreted normal amounts of tryptophane in the urine (16), but that excretion would appear to be abnormally high if the xanthurenic acid excreted, equivalent to 10 to 24 per cent of the tryptophane ingested (17), were also considered as excreted tryptophane. A high excretion of amino acids was reported in 1925 in urine from human subjects fed large amounts of wheat (18). This might parallel the present high excretion of amino acids by mice fed proteins of inferior biological value.

SUMMARY

1. Mice fed diets containing incomplete proteins (oxidized casein plus cystine and either tryptophane or methionine) excreted from 9.9 to 86.5 per cent of the ingested amino acids in the urine in microbiologically available form. The mean excretion was 24.5 per cent.

2. On complete proteins, such as casein, fibrin, or oxidized casein supplemented with cystine, tryptophane, and methionine, the different amino acids excreted ranged from 0.5 to 10.0 per cent, with a mean of only 2.9 per cent.

3. Urines from mice on either type of protein contained approximately the same amount of peptides. Relatively, however, a much higher per-

centage of the urinary amino acids was microbiologically available on incomplete proteins than when complete proteins were fed.

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THE RÔLE OF VITAMIN B₆ IN THE SYNTHESIS OF TRYPTOPHANE FROM INDOLE AND ANTHRANILIC ACID BY LACTOBACILLUS ARABINOSUS*

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Lactobacillus arabinosus has the ability to synthesize several amino acids when adequate quantities of vitamin B₆ are included in the medium (1, 2); however, the requirement of this organism for tryptophane is apparently not affected by the addition of vitamin B₆ (2). Earlier studies showed that indole and anthranilic acid have appreciable tryptophane activity for *L. arabinosus* (3–7) but not for *Streptococcus faecalis* R (3, 7). Serine enhanced the activity of indole for *L. arabinosus* when tested with a purified amino acid medium, but no increase in the tryptophane activity was noted when serine was added to a casein hydrolysate medium (7). Tatum and associates (8–10) have observed that serine is necessary for the synthesis of tryptophane from indole by certain *Neurospora* mutants, while Umbreit *et al.* (11) have shown that pyridoxal phosphate functions as a coenzyme for *Neurospora* in the synthesis of tryptophane from indole.

In the present study the influence of pyridoxine, pyridoxal, and pyridoxamine and other constituents of the medium on the biosynthesis of tryptophane from indole and anthranilic acid by *Lactobacillus arabinosus* was investigated.

EXPERIMENTAL AND RESULTS

A purified amino acid medium (12) and a casein hydrolysate medium supplemented with cystine (7) and lacking in tryptophane were used. In the present study synthetic *Lactobacillus casei* factor was also added to both media at a level of 0.02 γ per tube and *l*-proline was added to the purified amino acid medium at a level of 0.5 mg. per tube. In some tests the acetate buffer was replaced by a phosphate buffer (1 mM of KH₂PO₄ (2) and 10 mg. of sodium acetate (13) per tube). The microbiological tests were conducted by incubating the assays for 72 hours at 37° and the acid

production determined titrimetrically (bromothymol blue indicator). When the phosphate buffer was used, the titrations were carried out with a pH meter.

The tryptophane activity of indole and anthranilic acid was calculated from the tryptophane curves obtained with each assay. When variations in the basal media were made, a tryptophane curve was included with each change in the media and the activity of the compounds tested determined from the respective tryptophane curves. In all cases the activity of indole and anthranilic acid is expressed as the per cent tryptophane activity on a molar basis.

Influence of Pyridoxine, Serine, and Acetate—The first experiments were conducted to determine the effect of pyridoxine on the tryptophane activity of indole and anthranilic acid. Indole and anthranilic acid at concentrations of 2.5 to 10 γ per tube were used and the effect of adding 0 and 20 γ of pyridoxine hydrochloride determined. The influence of serine (1 mg. per tube of the *dl* isomer) tested with the acetate and phosphate buffers was also determined. The results of these experiments are shown in Table I.

It can readily be seen that pyridoxine has a marked effect on the ability of *Lactobacillus arabinosus* to utilize either indole or anthranilic acid as a source of tryptophane. With the purified amino acid medium and an acetate buffer, less than 5 per cent activity was observed for indole without pyridoxine and 91 per cent tryptophane activity with pyridoxine. The activity was appreciably higher when an acetate buffer was used compared to that observed with the phosphate buffer, suggesting that acetate as well as serine is involved in the synthesis of tryptophane. The higher activity obtained with the casein hydrolysate medium compared to that obtained with the purified amino acid medium substantiates an earlier observation (7).

The presence or absence of vitamin B₆ in the medium did not have any significant effect on the acid production with the tryptophane standard. Similarly, no effect of the presence or absence of serine could be detected with tryptophane.

In the absence of added pyridoxine, appreciable amounts of tryptophane were formed from indole and anthranilic acid with the casein hydrolysate medium, while little or none was formed when the purified amino acid medium was used. This suggested that small amounts of vitamin B₆ present in the casein hydrolysate could account for this difference in tryptophane activity. Furthermore in earlier work (7) only 2 γ of pyridoxine were used and a 25 per cent lower activity was observed for indole than was obtained in the present work with 20 γ of pyridoxine.

Detailed data from a typical experiment on the effect of graded levels of indole and anthranilic acid tested with an acetate buffer and a phosphate

buffer are shown in Table II.   The differences in the activity obtained with the two media are readily apparent.   Also, the activity tends to be lower at the higher test levels of indole and anthranilic acid with the phosphate medium.

TABLE I

Effect of Pyridoxine on Tryptophane Activity of Indole and Anthranilic Acid for Lactobacillus arabinosus

All values expressed as per cent tryptophane activity on a molar basis.

Medium used*	Indole		Anthranilic acid	
	Plus pyri-doxine†	No pyridoxine	Plus pyri-doxine†	No pyridoxine
A (acetate)	91	<5	33	<5
" (" + serine)	· 100	<5		
" (phosphate)	39	<5	23	<5
" (" + serine)	54	<5		
B (acetate)	105	41	66	28 .
" (phosphate)	85	41	43	25

* Medium A, purified amino acid medium, Medium B, casein hydrolysate medium plus cystine.

† 20 γ per tube of pyridoxine hydrochloride were used.

TABLE II

*Effect of Level of Indole and Anthranilic Acid and Buffer Used on Tryptophane Activity**

	Acetate buffer		Phosphate buffer	
	Indole	Anthranilic acid	Indole	Anthranilic acid
γ per tube				
2.5	110	65	98	56
2.5	105	69	98	48
5 0	103	66	86	40
5.0	102	67	86	40
7.5	100	55	75	42
7.5	98	60	77	41
10.0	100	59	72	36
10.0	100	56	80	38

* In these tests a casein hydrolysate medium plus cystine was used and 20 γ of pyridoxine hydrochloride were added per tube.

Comparative Effects of Pyridoxine, Pyridoxal, and Pyridoxamine—A series of experiments was conducted with indole and anthranilic acid in which pyridoxine was added at levels which ranged from 0 to 40 γ per tube. Indole and anthranilic acid were added at levels of 5 γ per tube and the

per cent tryptophane activity observed. The activity increased with higher levels of pyridoxine and reached a maximum with 15 to 20 γ of pyridoxine added per tube. Experiments were then conducted with pyridoxine, pyridoxal, and pyridoxamine and the results are shown in Table III. The latter two vitamin B₆ derivatives were much more active than pyridoxine, which is in agreement with their relative activities for other metabolic reactions (14, 15). Pyridoxamine was somewhat more active than pyridoxal. Similar tests conducted with anthranilic acid showed that

TABLE III

Relative Effects of Pyridoxine, Pyridoxal, and Pyridoxamine on Tryptophane Activity of Indole

An acetate buffer was used with the purified amino acid medium. 5 γ of indole were added per tube. Pyridoxal, pyridoxine, and pyridoxamine were added as the hydrochloride. The amounts indicated are not calculated back to the free base.

Pyridoxine	Tryptophane activity	Pyridoxal	Tryptophane activity	Pyridoxamine	Tryptophane activity
Autoclaved with medium					
γ per tube	per cent	γ per tube	per cent	γ per tube	per cent
0.5	10	0.005	7	0.005	49
1	13	0.01	14	0.01	72
2	28	0.02	63	0.02	99
5	51	0.05	75	0.05	114
10	72	0.1	83	0.1	116
20	87	0.5	113	0.5	107
40	85	1.0	107	1.0	116
Added to medium after autoclaving					
1	3	0.005	49	0.005	65
2	10	0.01	73	0.01	83
5	29	0.02	101	0.02	103
10	45	0.05	99	0.05	107
15	55	0.1	99	0.1	100
20	59				
40	64				

the comparative effectiveness of pyridoxine, pyridoxal, and pyridoxamine was approximately the same as that for indole.

As in the first experiments, occasionally a low activity (less than 10 per cent) was observed when no vitamin B₆ had been added. In these cases, this amount was subtracted from the value obtained at any level of vitamin B₆ derivative in order to express all data on a comparable basis.

Since pyridoxine has been shown to be converted to some extent to "pseudopyridoxine," now known as pyridoxal and pyridoxamine, by heat-

ing with amino and keto acids (16–19), other experiments were carried out in which the vitamin was sterilized separately and graded quantities added to the medium after autoclaving. As was expected, the effectiveness of pyridoxine was greatly reduced (Table III). ˙Pyridoxal and pyridoxamine were more active, which would suggest that either they are converted to some extent to pyridoxine during autoclaving or are partially inactivated. These data offer further evidence that pyridoxal and pyridoxamine function most effectively in the transformation of indole to tryptophane.

The activity observed for indole was frequently over 100 per cent of the theoretical. This suggests that *Lactobacillus arabinosus* has a limited capacity to synthesize the indole nucleus under certain conditions. This observation has also been reported by others (4, 5). The variability in tryptophane activity of indole reported by various investigators (3–7) can apparently be explained by the fact that different levels of pyridoxine were used in the basal media.

<div align="center">DISCUSSION</div>

These studies demonstrate another metabolic process in which vitamin B_6 functions. Umbreit and associates (11) have recently demonstrated a marked increase in the activity of a cell-free preparation of *Neurospora* for the transformation of indole to tryptophane when pyridoxal phosphate was added. Thus, vitamin B_6 appears to function in a similar manner for both *Lactobacillus arabinosus* and *Neurospora* in this synthesis.

The actual pathways by which anthranilic acid and indole are converted to tryptophane are unknown. The effects of serine and acetate suggest that they may serve, at least in part, as constituents of the side chain of tryptophane. Lyman (2) has shown that acetate and CO_2 favor the synthesis of other amino acids by *Lactobacillus arabinosus*. The effect of other amino acids, such as glycine, as well as the effect of CO_2 may afford additional information on the pathways of tryptophane synthesis from indole and anthranilic acid by this organism.

<div align="center">SUMMARY</div>

1. Vitamin B_6 is necessary for the metabolic conversion of indole and anthranilic acid to tryptophane by *Lactobacillus arabinosus*.

2. Pyridoxal and pyridoxamine are much more active than pyridoxine for this transformation. The influence of pyridoxal and pyridoxamine was increased when added to the medium after autoclaving, while that of pyridoxine was decreased.

3. Serine and acetate appear to increase the activity of indole and anthranilic acid.

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THE COMPOSITION OF FLAXSEED MUCILAGE*

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Flaxseed mucilage contains d-galacturonic acid, l-rhamnose, l-galactose, and d-xylose. On partial hydrolysis it yields an aldobionic acid in which the d-galacturonic acid is attached by a 1:2 linkage to l-rhamnose (1–7). Neville (2) prepared the mucilage in yields as high as 6.3 per cent. He described it as a white powder with $[\alpha]_D^{25} = +10.79°$, giving by alkaline titration an equivalent weight of 710. He reported that the ash contained calcium, potassium, magnesium, iron, and phosphorus. Tipson, Christman, and Levene (6) found that when the viscous mucilaginous solution is heated for a short time in a boiling 1 per cent solution of hydrochloric acid the viscosity decreases greatly and the solution can be filtered rapidly through cloth. They prepared the ash-free mucilage by this procedure.

This investigation is an attempt to determine the percentages of the various constituents in the mucilage and the order in which they are liberated during hydrolysis.

EXPERIMENTAL

Preparation of the Mucilage—The unchanged mucilage was prepared from freshly cleaned flaxseed as described by Neville (2, 7). When the thick extract was poured into 5 volumes of ethanol without stirring, a white fibrous mass soon rose to the surface, leaving the heavier impurities below. This mass was lifted out and added to fresh ethanol and later filtered at the pump. It was washed with ethanol and ether and dried rapidly *in vacuo.*

The ash-free mucilage, or linseed acid, was prepared rapidly and easily directly from flaxseed by a slight modification of the procedure of Tipson, Christman, and Levene (6). Clean flaxseed was mixed in a large flask with 4 times its weight of water and allowed to stand for 24 hours. The mixture was then heated in a bath of boiling water until the temperature inside the flask was 80°. Sufficient concentrated hydrochloric acid, mixed with 2 volumes of water, was then added to make the solution 2 per cent acid and the heating at 80° was continued for 3 minutes. The hot solution was filtered rapidly, at the pump, through several layers of cloth and the seed was washed twice with water. The filtrate was immediately added

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to 5 volumes of ethanol and the product isolated as previously described. The yield was 5.8 per cent of white, fluffy linseed acid. On standing, an additional 0.4 per cent settled out of the alcohol. However, it was higher in ash and poorer in quality than the main preparation.

Analysis of Flaxseed Mucilage and Its Acid—The mucilage and its free acid were analyzed for ash, uronic acid anhydride, xylan, rhamnosan, galactan, $[\alpha]_D^{25}$, and the equivalent weights were calculated from the per cent uronic anhydride and determined by alkaline titration of the free acid. The results of the analyses are given in Table I. Most of the determinations were made by standard methods and need not be described. Xylan and rhamnosan were determined from the phloroglucides by the method of Tollens and Ellett (8). Many investigators have been unable to obtain satisfactory results with this method. It does not seem sound theoretically, since it is based on the assumption that mixed phloroglucides of furfural and methyl furfural are not formed when a mixture of the two furfurals is treated with phloroglucinol. The formation of plastics by phenols and various furfurals suggests that this assumption may not be correct (9). In the present investigation extraction of the phloroglucides with 95 per cent ethanol in the Tollens-Ellett procedure left large amounts of rhamnosan phloroglucide undissolved. This would be expected if mixed phloroglucides of the two furfurals, similar to plastics, were formed when phloroglucinol is added to a mixture of the two furfurals. When the galactan in linseed acid and the salts of the aldotrionic acids were determined by oxidation to mucic acid with nitric acid as directed by van der Haar (10), exactly 1 mole of galactan was in each case obtained for each equivalent weight of the acid that was being oxidized. When the per cent galactan was calculated by difference between 100 and the sum of the other percentages, it agreed closely with the experimental determination. The mucic acid must come in part from *d*-galacturonic acid and in part from the *l*-galactose since it is known that neither of these, when in combination, is oxidized quantitatively by nitric acid to mucic acid.

Comments on Table I—The results are calculated to the ash-free basis.

During the investigation many preparations of the mucilage and the free acid were analyzed. These materials cannot be purified in the usual sense of the word. When they are hydrolyzed by heating with a dilute acid, varying amounts of an insoluble residue remain. This consists in part of polymerized furfural and in part of materials that passed into colloidal solution during extraction of the seed and were precipitated with the mucilage. The equivalent weight of different preparations of the mucilage varied considerably, depending on how clean the seed was and how much of this foreign material was present. The high equivalent weight, 731, of this sample of mucilage is due to the presence of these impurities. Much

of this foreign material is removed by conversion to the free acid, since the equivalent weight of the acid, 639, is near the theoretical equivalent weight, 634. If the percentages of uronic acid and xylan plus rhamnosan, 26.4 and 39.6, in the mucilage are corrected for this foreign material, they become 30.46 and 45.66, which approximate the theoretical percentages of the free acid.

Difficulty was met in getting checks in the uronic acid termination both of the mucilage and of the free acid.

TABLE I

Analysis of Flaxseed Mucilage and Its Free Acid

	Mucilage		Free acid	
	Found (a)	Found values calculated to free acid* (b)	Found	Theory
Ash, %	0.87		0.91	0.00
CO_2, %	6.02	6.94	6.88	6.94
Uronic acid, %	26.40	30.46	30.33	30.60
Xylan, %	27.20	31.36	32.55	20.82
Rhamnosan, %	12.40	14.30	11.75	23.03
Xylan + rhamnosan, %	39.60	45 66	44 30	43.85
Equivalent weight from CO_2	731	634	639.00	634.00
" " by titration			653.00	634.00
$[\alpha]_D^{25}$, degrees	+10 78†	+12.08	+11.9	
Insoluble residue, %			1.76	0
Galactan by difference, %		23.88	23.61	25.55
" " mucic acid, %.			29.30	25.55

* The data in column (a) multiplied by 731/634 give the values in column (b).

† This value was reported by Neville (2) for a mucilage with an equivalent weight 710

The per cent xylan was always much higher and of rhamnosan much lower than the theory. However, the sum of these two percentages found checked closely the theoretical sum of the two. It may be that when phloroglucinol is added to a mixture of the two furfurals mixed phloroglucides are formed which cannot be separated by extraction with 95 per cent ethanol. Since, as will be shown later, the aldotrionic acid formed by partial hydrolysis of the mucilage contains 1 mole of rhamnosan for each equivalent weight and approximately 1 mole of xylan was hydrolyzed off, it appears that in the linseed acid the xylan and rhamnosan are present in molecular proportions.

The mucilage is chiefly the calcium salt of linseed acid. Its equivalent weight should be 654. The $[\alpha]_D^{25}$ found for the mucilage was reported by

Neville to be $+10.78°$ and the equivalent weight 710. This would correspond to $[\alpha]_D^{25} = +12.08°$ for the linseed acid, which was found experimentally to be $+11.9°$.

Since crystalline d-xylose, l-galactose, and l-rhamnose were repeatedly obtained from the sugar syrups and no evidence of any other sugars was obtained, the results summarized in Table I, together with those given later in Table II, indicate strongly that the ash-free mucilage or linseed acid is composed of molar equivalents of d-galacturonic acid and each of the above three sugars.

Tests for Other Sugars—Non-crystalline sugar syrups obtained after heating the mucilage for 10 hours with a 4 per cent solution of sulfuric acid and removal of some crystalline d-xylose and l-galactose did not ferment when treated with ordinary yeast and gave no mannose phenylhydrazone when treated with phenylhydrazine in the cold. These syrups still contained large amounts of d-xylose and l-galactose as shown by the Bertrand and mucic acid tests. Also the non-crystalline syrup remaining after isolation of l-galactose from the aldotrionic acid showed no fermentation with ordinary yeast but did give the mucic acid test for galactose. The common sugars fermentable by ordinary yeast are thus absent. No positive evidence of any sugar, except the three already described, has been obtained.

Hydrolysis of Mucilage—Previous work (3) has shown that l-rhamnose is attached directly to the d-galacturonic acid and is the last sugar liberated during hydrolysis of the mucilage. To determine which sugar is first liberated the mucilage was fractionally hydrolyzed and the free sugar separated from the resulting aldotrionic acid. During these fractional hydrolyses it was not possible to liberate all of the first sugar that is freed and none of the second sugar. Mixtures of sugar acids result, some containing all three sugars, some with two sugars attached, and some with only the l-rhamnose. Furthermore when a bivalent metal such as barium is used, mixed salts containing two different acid radicals attached to the same positive ion are precipitated by addition of alcohol. After many fractional hydrolyses, the following procedures showed that d-xylose is the first sugar liberated and that the aldotrionic acid consists of d-galacturonic acid, l-rhamnose, and l-galactose.

Hydrolysis with Sulfuric Acid and Isolation of d-Xylose—At different times several lots of linseed acid were dissolved in 24 times their weight of a 4 per cent solution of sulfuric acid and heated to $80°$ for 4 hours. After neutralization with barium carbonate, the solutions were filtered from barium sulfate and the filtrates were concentrated *in vacuo*. The barium salts were separated from the sugar by precipitation with alcohol. The salts were purified by dissolving in water and reprecipitating with alcohol.

The alcohol solutions of the sugars were concentrated *in vacuo* and freed of small amounts of salts by addition of alcohol. When the sugar syrups were dissolved in glacial acetic acid, crystalline *d*-xylose was obtained in all cases. On analysis, the barium salts showed equivalent weights, calculated from the per cent carbon dioxide, that varied between 510 and 550. Since the equivalent weight of the barium salt of an aldotrionic acid consisting of *d*-galacturonic acid, *l*-rhamnose, and *l*-galactose should be 570, it is evident that all of the *d*-xylose and some of the *l*-galactose had been freed.

For example, in one case 50 gm. of linseed acid were hydrolyzed and the salts and sugar syrup were isolated. On analysis the sugar syrup was found to contain 8.76 gm. of xylan and 0.878 gm. of galactan and no rhamnosan. These weights correspond respectively to 17.5 and 1.75 per cent of the original linseed acid. Since this acid contained approximately 21 per cent xylan and 25 per cent galactan, it is evident that most of the xylan and a small part of the galactan were free in the sugar syrup. On analysis, the barium salt gave no xylan, 29.2 per cent rhamnosan, 8.58 per cent carbon dioxide, and had an equivalent weight of 518.

Hydrolysis with Hydrochloric Acid and Preparation of Aldotrionic Acid— Numerous hydrolyses of the linseed acid with a 2 per cent solution of hydrochloric acid showed that heating for 1 hour at 80° gave an aldotrionic acid composed of *d*-galacturonic acid, *l*-rhamnose, and *l*-galactose. In these hydrolyses the linseed acid was dissolved in 24 times its weight of water and pressed through cloth to remove any lumps. The solution was then heated to 80°. Sufficient concentrated hydrochloric acid to make the solution 2 per cent acid was mixed with twice its volume of water and heated to 80°. The two solutions were mixed and the heating at 80° was continued for 1 hour. The solution was then rapidly neutralized. In some experiments sodium hydroxide solution was used to neutralize the acids. In other experiments a hot strontium hydroxide solution was used for this purpose. The latter is the more satisfactory, since the strontium salts are less soluble and require less alcohol for precipitation. The salts were precipitated by addition of the solution to alcohol. The corresponding sodium and strontium salts of the aldotrionic acid were freed of chlorides by washing with 85 per cent alcohol. The results obtained on analysis of these salts are given in Table II.

When the filtrates from the preparation of the aldotrionic acids were analyzed, approximately 18 per cent of the weight of the linseed acid used appeared as xylan and 1.5 per cent as galactan. No rhamnosan was found in the filtrates. Since these percentages correspond to most of the xylan present in the linseed acid and only a small part of the galactan, the results indicate that *d*-xylose is the first sugar liberated.

Comments on Table II—The theory is calculated for the salts of an aldotrionic acid composed of *d*-galacturonic acid, *l*-rhamnose, and *l*-galactose. All of the xylan should have been hydrolyzed off. However, in the rhamnosan determination some of the phloroglucides did not dissolve and there probably were small amounts of xylan present. This could have been furfural from the galacturonic acid.

The galactan determination was carried out by the van der Haar method. The results have been discussed.

In general the barium or strontium salts of sugar acids with a free sugar group give high values for the metal. It may be that the sugar group, possibly in the enolic form, reacts with some of the hydroxide. This would explain the high percentage of strontium in the salt.

TABLE II

Analyses of Sodium and Strontium Salts of Aldotrionic Acid from Hydrolysis of Linseed Acid

	Sodium salt		Strontium salt	
	Found	Theory	Found	Theory
Carbon dioxide, %	8.39	8.40	8.10	8 08
Uronic acid, %	36.80	36 84	35 53	35 45
Equivalent weight.	524	524	543 0	545
Rhamnosan + xylan, %..	27.45	27.86	28 50	26.78
Galactan, % .	31.50	30 9	31 80	29 72
Sodium or strontium, %..	4.25	4.39	9 20	8.03
$[\alpha]_D^{25}$, *degrees* .	+62.0		+60	

The great increase in dextrorotation of the linseed acid during hydrolysis is probably due in part to depolymerization of the complex molecule rather than simply to the liberation of *d*-xylose.

Hydrolysis of Aldotrionate and Isolation of l-Galactose—36 gm. of strontium aldotrionate were heated for 4 hours in a bath of boiling water with 1 liter of 4 per cent sulfuric acid. The solution was neutralized with barium carbonate, decolorized with carbon-black, and concentrated *in vacuo* to 200 cc. The concentrate was added slowly to 6 volumes of alcohol and the barium aldobionate was filtered off, redissolved in water, and again precipitated by ethanol. The ethanol sugar solution was concentrated *in vacuo* to a small volume and taken up in ethanol to remove small amounts of barium salts. The gum sugar finally obtained weighed 11 gm. It was stirred in a bath of boiling water with 1 cc. of water. Then 6 cc. of glacial acetic acid were added and the stirring was continued until all dissolved. The solution was cooled and seeded with *l*-galactose. In a short time the solution became solid with crystals. The first crop weighed 2.5 gm. and

the second crop 0.25 gm. The filtrate from the second crop was diluted
with ethanol. This precipitated some gummy barium salt. After con-
centrating the filtrate, a third crop of l-galactose was obtained which
weighed 0.25 gm. The total yield of crystalline l-galactose was 8.3 per
cent. The first crop of crystals, after addition of 1 drop of ammonia,
showed $[\alpha]_D^{25} = -78.8°$. On oxidation with nitric acid this gave mucic acid,
melting at 216°. This hydrolysis of strontium aldotrionate has been re-
peated many times, always with approximately the same results. The
yield of crystalline l-galactose varies from 5.5 to 8.5 per cent of the aldo-
trionate used. The theoretical yield should be 33 per cent. There is
certainly considerable loss of the sugar in the precipitates and during decol-
oration and some remains in the non-crystalline syrups.

In one case in which the sugar stood some days with glacial acetic acid
before seeding, $1\frac{1}{2}$ minutes after solution the first crop of crystals showed
$[\alpha]_D^{25} = -120°$ and 2 minutes after solution $-110°$. In approximately
10 minutes $[\alpha]_D^{25} = -90°$. When a drop of ammonia was added $[\alpha]_D^{25} =$
$-78.8°$. This mutarotation indicates that the sugar, under these con-
ditions, crystallizes in the α form, $[\alpha]_D^{25} = -145°$.

The preparation of l-galactose as described by Anderson (4) often yields
chiefly d-xylose because the two sugars are present in the hydrolysate.
This difficulty can be avoided by first preparing the strontium salt of the
aldotrionic acid, thus removing the d-xylose, and afterwards hydrolyzing
the aldotrionic acid and isolating the l-galactose. In this preparation it is
not necessary to isolate the mucilage. The flaxseed is extracted for 3
minutes at 80° with a 2 per cent solution of hydrochloric acid. The hot
solution is filtered from the seed and the heating at 80° is continued for
55 minutes. The hot solution is carefully neutralized with a hot strontium
hydroxide solution and the strontium aldotrionate is precipitated by addi-
tion to 6 volumes of ethanol. It is then purified and the l-galactose
liberated.

*Heating Linseed Acid to 80° for 3 minutes in 2 Per Cent Solution of Hy-
drochloric Acid*—10 gm. of linseed acid were dissolved in 700 cc. of water
and the solution was heated to 80°. To this were added 300 cc. of a solution
containing 45 cc. of concentrated hydrochloric acid which had been heated
to 80°. The heating at 80° was continued for 3 minutes. At this time the
solution had lost its high viscosity and could be poured rapidly through
cloth. It was cooled in an ice bath to 30° within 3 minutes and sodium
hydroxide solution was carefully added until the solution was slightly
acid. At this time the solution was viscous, like flaxseed mucilage. It was
poured into 12 volumes of ethanol and the flocculent precipitate was iso-
lated and washed with 85 per cent ethanol until free of chlorides. A
carbon dioxide determination showed an equivalent weight of 670. The

alcohol filtrate was concentrated *in vacuo*. Pentosan and uronic acid determinations showed that it contained no more than traces of xylose or galacturonic acid. Evidently the mucilage undergoes no appreciable change in composition under these conditions.

Structure of Flaxseed Mucilage—Aside from the presence of a 1:2 linkage between the *d*-galacturonic acid and *l*-rhamnose, little is known of the structure of the unchanged mucilage. It seems to be composed of a series of polymerized molecules of an aldotetronic acid containing *d*-galacturonic acid, *l*-rhamnose, *l*-galactose, and *d*-xylose. It does not reduce Fehling's solution. Its physical properties resemble those of starch to a considerable extent. This suggests that it may have some form of a branched chain structure. While *d*-xylose is the first sugar liberated, this does not imply that the three sugars form a chain with the *l*-galactose in the middle. The repeating unit may be the aldobionic acid with the *l*-galactose and *d*-xylose as side chains or it may be the aldotrionic acid with the *d*-xylose attached as a side chain in the furanose form. Work is now in progress on the oxidation of the aldotrionic acid to a dibasic acid which should throw further light on the structure of the mucilage. Probably the first step in the hydrolysis of the mucilage consists in depolymerization of the large aggregate because heating to 80° in a 2 per cent solution of hydrochloric acid for some time is necessary before appreciable reduction of Fehling's solution occurs.

SUMMARY

1. A rapid method for preparing the free acid of flaxseed mucilage, linseed acid, directly from the seed is described.

2. Analysis of flaxseed mucilage and linseed acid shows that they are composed of molar equivalents of *d*-galacturonic acid, *l*-rhamnose, *l*-galactose, and *d*-xylose.

3. During hydrolysis of the mucilage *d*-xylose is the first and *l*-galactose the second sugar to be liberated.

4. An aldotrionic acid composed of molar equivalents of *d*-galacturonic acid, *l*-rhamnose, and *l*-galactose has been isolated.

5. An improved method for preparing *l*-galactose from the mucilage is described.

6. Flaxseed mucilage seems to be the salt of a polymerized aldotetronic acid composed of molar equivalents of *d*-galacturonic acid, *l*-rhamnose, *l*-galactose, and *d*-xylose. The physical properties of the mucilage suggest that it has a branched chain structure.

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THE ESTIMATION OF BASIC ORGANIC COMPOUNDS IN BIOLOGICAL MATERIAL

I. GENERAL PRINCIPLES*

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Simple and reliable methods for the measurement of the concentration of therapeutic agents in biological materials greatly facilitate the study of the agent's physiological disposition, upon which sound therapy must be based. The intermediate metabolism of a therapeutic agent cannot be studied in the absence of methods for its estimation. And finally, knowledge of the concentration of an agent in the tissues and fluids of the host, animal or human, is of considerable value in studying the mechanism of drug action. Unless the concentration *in vivo* of an agent, achieved in a specific tissue after therapeutic dosage, is known, the interpretation of studies *in vitro* with the agent is open to question.

The problem of devising analytical procedures for the estimation of a large number of compounds, in connection with an antimalarial clinical testing program, was simplified by a scheme for the analysis of basic organic compounds. The principles involved in this scheme are presented here and should be applicable to the development of methods for other basic therapeutic agents. The various methods, with examples to illustrate their sensitivity and specificity, will be described in subsequent papers.

The scheme involves the isolation of the compounds from their metabolic products and from normally occurring substances by extraction procedures. The concentration of the compound is increased if necessary by returning it to a small aqueous phase. The method for the final estimation is determined by the properties of the compound and depends on fluorometry or photometry. A general technique of appraising specificity is based on a comparison of the solubility characteristics of the pure compound with those of the material extracted from the biological material obtained from a host to which the drug has been administered. Data obtained from this comparison may be used to modify the initial extraction procedure so as to separate interfering drug metabolites from the unchanged drug.

The application of this systematic approach to a basic organic compound includes a number of discrete steps which are described below.

Selection of Method of Assay

The simplicity and speed of fluorometric assay recommend it as the method of choice when possible. The successful application of this technique is limited, however, by the available mercury arc instruments which confine the wave-length of the activating radiation to the 365, 405, and 436 mμ lines. Compounds whose fluorescence may be activated by light of other wave-lengths cannot be measured with these instruments.

Some compounds can be converted to fluorophores by suitable treatment, such as oxidation, hydrolysis, or ultraviolet irradiation. Other methods of producing fluorescent compounds may also be possible. This approach has been found especially favorable when the compound in question has an absorption maximum above 300 mμ. A nuclear change in the molecule may then be accompanied by a shift of its absorption spectrum towards the higher wave-length and into the range of one of the mercury arc emission bands.

The fluorescence of the compound in water is measured at various pH levels to determine the conditions of maximal sensitivity. To determine whether the compound will fluoresce with sufficient sensitivity in an organic solvent, the compound is extracted into the selected solvent (see later) and its fluorescence measured after the addition of 0.1 volume of a 25 per cent solution of trichloroacetic acid in ethylene dichloride.

If fluorescence techniques cannot be applied, a coupling reaction to form a dye is next tried. Aromatic amines may usually be diazotized and coupled with N-(1-naphthyl)ethylenediamine (1). Substituted aromatic amines, if the position para to the amino group is free, may usually be coupled with diazonium salts to form colored azo compounds (2). Certain primary amines, for instance 2,6-diaminopyridine, which diazotize with difficulty, may be coupled with diazonium salts. The above reactions are not subject to interference from organic extractable substances normally occurring in biological material. Consequently, high sensitivity may be achieved by making the final photometric measurement in a small volume.

Most organic bases form methyl orange salts, which are highly soluble in certain organic solvents, but relatively insoluble in water. Bases may, therefore, be assayed indirectly through the extraction of their methyl orange salts into an organic phase. The methyl orange which goes into the organic phase is measured photometrically (3). This general reaction has been used in the design of analytical procedures for a large number of organic bases. Here, too, in many cases, the final photometric measurement

may be made in a small volume. The sensitivity of this reaction is limited, however, by the interference of a small amount of naturally occurring substances in the plasma.

Although spectrophotometry in the ultraviolet region lacks the sensitivity of the methods of assay previously described, it is useful as a final means of assay in certain instances, and as an aid in developing procedures which eventually involve other principles of assay. The absorption spectrum of the compound in 0.1 N H_2SO_4 is investigated to determine the wave-length of the absorption maximum most suitable for quantitative measurements. A basic compound is usually suited for measurement in the ultraviolet if 1 γ per ml. results in an optical density of 0.050 or more at some wave-length above 240 mμ.

Extraction of Compound from Aqueous Solution

The solvents that have been used in the procedures for basic compounds are, in order of diminishing polarity, ethylene dichloride, benzene, and petroleum ether or heptane. Technical grades of these solvents have been found quite satisfactory provided that contaminants are first removed by acid and alkaline washes. Most petroleum ethers have been found to be unsuitable when the fluorescence measurement is made directly in this solvent because of a high fluorescence blank. Heptane, however, may be used in these instances.

The adsorption of compounds from organic solvents, by glass surfaces, may constitute a major source of error in the procedures described here because of the micro amounts involved. The extent of this adsorption depends on the properties of the compound and on the polarity of the solvent, the adsorption being greatest from the less polar solvents. The loss is minimized by the addition of an alcohol to the solvent before or subsequent to the initial extraction. The extent of adsorption of chloroquine (7-chloro-4-(4-diethylamino-1-methylbutylamino)quinoline) from various solvents, with and without the presence of ethanol is shown in Table I. The data indicate that more of this compound is adsorbed from heptane, the least polar solvent, and that ethanol minimizes this adsorption. Some organic bases also are adsorbed on glass from neutral or alkaline aqueous solutions. For this reason standards are routinely made up in acid solution. The solvent of choice is the one which will extract the compound quantitatively with the least amount of its interfering metabolites. To select the solvent and define the pH for the extraction, the fraction of the compound extracted from water is measured at various pH values (1 to 14) with each solvent. A simple way of doing this is to extract relatively large amounts of the drug (in the order of 10 γ per ml.) with equal volumes

of solvent and measure the unextracted residue by ultraviolet absorption. The solvent of choice is, as is shown later, the least polar one which will extract the compound quantitatively.

Recoveries of Known Amounts of Compound from Aqueous Solution—Known amounts of the compound (in the range of 0.25 to 10 γ) are extracted with the selected solvent and estimated by the method of assay found to be the most suitable. The amount of compound to be carried through the procedure will depend on the sensitivity of the final measurement. The exact conditions of the procedure will depend of course on the solvent used and on the final method of assay. These conditions are described in the subsequent papers of this series.

TABLE I

Adsorption of Chloroquine on Glass from Various Organic Solvents

Duplicate 10 γ samples of chloroquine were extracted from alkaline solution into 150 ml. of solvent. After separation of the phases, 2 ml. of ethanol were added to the organic phase of one sample, the other being used as a control. The aqueous phase was discarded, and 15 ml. aliquots of solvent were removed at intervals and analyzed for chloroquine. The recovery of chloroquine at zero time is arbitrarily designated as 100 per cent.

| | Per cent recovery of chloroquine | | | | | |
| | From ethylene dichloride | | From benzene | | From heptane | |
Time	Without ethanol	With ethanol	Without ethanol	With ethanol	Without ethanol	With ethanol
min.						
0	100	100	100	100	100	100
5	98	101	96	100	94	97
15	99	100	100	104	81	104
30	97	98	100	106	76	103
60	100	99	106	100	75	97

Extraction of Compound from Biological Material

Plasma[1]—The handling of the plasma in the extraction procedure varies somewhat with the solvent used. As a rule, with ethylene dichloride, the

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[1] Certain drugs, such as quinacrine and chloroquine, are greatly concentrated in the leucocytes. Therefore, special precautions in the preparation of plasma for analysis are required. Potassium oxalate is used as the anticoagulant. The use of ammonium oxalate must be avoided because exchange of the ammonium ion with the drug concentrated in the leucocytes may result in erroneously high plasma values (4, 5). The blood is centrifuged immediately, at 1500 R.P.M. for 30 minutes, and the plasma is separated carefully from any solid residuum. This procedure is deemed advisable in order to remove any possibility of contaminating the plasma sample with leucocytes or leucocyte fragments.

plasma (up to 10 ml. volume) is alkalized with 1 ml. of 2.5 N NaOH. 10 minutes of shaking in a glass-stoppered bottle are usually sufficient for complete extraction. The phases are separated by transferring the contents of the bottle to a test-tube, centrifuging at 3000 R.P.M., and removing the aqueous phase by aspiration. The larger volumes of plasma, when extracted with ethylene dichloride, sometimes result in the formation of a gel in the organic phase. The formation of these gels is minimized if the ethylene dichloride is added first to the bottle and the plasma carefully layered on the solvent without preliminary mixing. This prevents the formation of air bubbles at the interface of the two liquids.

With benzene as the solvent the procedure is the same as with ethylene dichloride except that it is not necessary to transfer to a tube for centrifugation, as the two phases usually separate easily or, if necessary, the bottle and contents may be centrifuged directly.

When heptane or petroleum ether is used, the plasma is diluted with an equal volume of 0.1 N NaOH and the mixture is shaken for 30 minutes. Dilution is necessary, since the plasma otherwise becomes quite viscous. This may result in incomplete extraction of the base because of the inefficient mixing of the two phases. When these solvents are used, the two phases separate easily, though again it is occasionally necessary to centrifuge the bottle and contents.

Certain organic bases may be determined directly in plasma filtrates after protein precipitation. This type of procedure has been described previously for the fluorometric determination of quinine (6). It has the advantages of speed and simplicity, but it lacks specificity, since interfering metabolic derivatives of the compound are not removed. In certain cases this method may be used routinely as an approximation procedure, provided the magnitude of error involved has been assayed by comparison with an extraction procedure of known specificity.

Tissues—Up to 2 gm. of tissue are added to 5 ml. of 0.1 N HCl and ground to an emulsion in an electrically driven homogenizer. This homogenizer consists of a glass cylindrical cup in which a close fitting ground glass pestle is mechanically rotated.[2] Cell fragmentation is complete. The emulsion, diluted if necessary with water, is transferred with a pipette prior to its extraction.

Feces—20 ml. of concentrated HCl are added to the feces and the whole diluted to 2 liters. The mixture is shaken on an automatic shaker until a homogeneous suspension results. The emulsion is then ready for extraction.

Urine—The urine is handled in the same way as plasma.

[2] A satisfactory device is distributed by the Scientific Glass Apparatus Company Bloomfield, New Jersey.

Recoveries of Known Amounts of Compound from Biological Material—
Known amounts of the compound are equilibrated with plasma for several
hours.   Aliquots of the plasma are analyzed in the same way as the aqueous
solutions described above.   Occasionally the recoveries from plasma are
incomplete, even though recoveries from water under similar conditions are
quantitative.   This is due to binding on plasma constituents.

The following should be tried.   (*a*) The plasma is adjusted to the lowest
pH which has been shown to allow complete extraction of the compound
from water.   The binding of organic bases on plasma constituents is
minimized at the lower pH values.   For instance, a large series of dialkyl-
aminomethylmethanol derivatives of phenanthrene, *e.g.*, SN 1796[3] (*α*-(di-
amylaminomethyl)-9-(1,2,3,4-tetrahydrophenanthrenemethanol)) can be
extracted quantitatively from alkaline aqueous solutions but not from
alkaline plasma.   However, the salts of these bases are soluble in organic
solvents, and, since the plasma binding is reversed in an acid medium, com-
plete extraction is achieved from acidified plasma.   (*b*) Longer shaking,
up to 1 hour, may result in complete extraction.   (*c*) The extraction is
tried with a more polar solvent.   The resulting loss of specificity may be
corrected, if necessary, by returning the compound from the solvent to
acid, from which the compound may be extracted, after alkalization, into
a less polar solvent.

After adequate recoveries of the compound from biological material are
achieved, the next step is to determine whether the method is sensitive
enough for estimation of the tissue levels after a prescribed dose.   The drug
is administered in the required dosage and the tissue concentrations deter-
mined.   If the amounts in a particular tissue are too small for estimation,
the procedure must be revised accordingly.   .

Assay of Specificity

The choice of the proper solvent is important in determining the speci-
ficity of an analytical procedure.   Experience has shown that, as a rule,
basic organic compounds are metabolized in the body to substances more
polar than the parent compounds.   They have, therefore, lower distribu-
tion coefficients between organic solvents and water than the parent drugs.
This solubility differential may be exploited to facilitate their separation by
choosing for the extraction the least polar solvent which quantitatively
extracts the parent drug.   Either all or a considerable fraction of the rela-
tively polar metabolic products may then be left behind in the initial

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[3] The SN number refers to the number assigned to the compound by the Survey of
Antimalarial Drugs, conducted in behalf of the Committee on Medical Research,
Office of Scientific Research and Development, and the Board for the Coordination
of Malarial Studies.

extraction, and the remainder removed by suitable aqueous washes of the solvent extract. Also, in those procedures in which a biological blank interferes in the final reaction, the blank is minimized by the use of a solvent with as low a polarity as possible.

Examples demonstrating the importance of the choice of the proper solvent are shown in Table II. Quinine is metabolized in the human to a derivative which contains an additional oxygen in the quinuclidine ring. The fractions of quinine and its metabolic derivative extracted from aqueous solution by various solvents at different pH values are compared in Table II. The data indicate that the least polar solvent which achieves complete extraction of quinine is benzene at pH 10 or higher. On the other

TABLE II

Distribution of Quinine and One of Its Metabolic Derivatives between Water and Solvents at Various pH Values

The fraction of the compound extracted at various pH values is expressed as the ratio of the amount of compound in the organic phase to total compound after shaking the buffered solution with an equal volume of solvent.

pH	Petroleum ether-water system		Benzene-water system		Ethylene dichloride-water system	
	Quinine	Quinine metabolic product	Quinine	Quinine metabolic product	Quinine	Quinine metabolic product
6	0.02	0	0.12	0	0.31	0
7	0.04	0	0.36	0	0.80	0
8	0.05	0	1.00	0.06	0.98	0
9	0.13	0	1.00	0 10	1.00	0.27
10	0.26	0	1 00	0.14	1.00	0.31
11	0.31	0	1.00	0.13	1 00	0.37
14 (1 N NaOH)	0.28	0	1.00	0.14	1.03	0 29

hand, the extraction of the metabolic derivative is relatively slight with benzene, though appreciable with ethylene dichloride. It may be concluded from these results that benzene is the solvent of choice for the extraction of quinine, since it quantitatively extracts quinine with little extraction of the metabolic product. Furthermore, the data indicate that the fraction of the metabolic product which does pass into the benzene may be removed by aqueous alkaline washes.

A further example of the importance of the proper choice of solvent in determining the specificity of a method is shown in the case of chloroquine. Chloroquine is metabolized in the human to derivatives which are present in plasma. These have been separated from plasma but have not been isolated in the pure state. The solubility characteristics of chloroquine and its metabolic derivatives are compared in Table III. The data indicate

that both chloroquine and its metabolic derivatives are extracted completely from aqueous solution by ethylene dichloride between pH 9 and 13 and that, therefore, no separation of the compounds may be made with this solvent. But with heptane, a less polar solvent, a separation is made at the higher pH. Here a portion of the metabolic derivatives remains behind in the initial extraction. The rest may be removed by washing the solvent extract with alkali.

The examination of the specificity of a method has been discussed in a previous paper (3) and the principles need not be repeated here. The technique has been modified so that smaller amounts of drug may be handled. The amounts required for the procedure described below range between 3 and 20 γ, depending on the sensitivity of the final measurement.

TABLE III

Distribution of Chloroquine and Its Metabolic Derivatives between Water and Solvents

The fraction of the compounds extracted at various pH values is expressed as the ratio of the amount of compound in the organic phase to total compound after shaking the buffered solution with an equal volume of solvent

pH	Heptane-water system		Ethylene dichloride-water system	
	Chloroquine	Chloroquine metabolic products	Chloroquine	Chloroquine metabolic products
9	0.70	0 25	1.00	1.00
14 (1 N NaOH)	1.00	0.51	1.00	1.00

The revised technique for assaying specificity was applied to chloroquine as follows: A volume of plasma from a patient receiving chloroquine, containing about 5 to 10 γ of apparent drug, was shaken for 1 hour with 6 volumes of heptane and 1 volume of 0.1 N NaOH. After the phases had settled, 1 ml. of ethyl alcohol for each 75 ml. of heptane was mixed with the heptane phase so as not to disturb the aqueous phase. As much as possible of the solvent was separated and the drug returned to 10 ml. of 0.1 N HCl. An aliquot of this solution was assayed for chloroquine and on the basis of this assay the solution diluted to contain about 0.5 γ of drug per ml. Two 1 ml. aliquots of the solution were set aside for direct analysis. 1 ml. aliquots of the solution were neutralized by the addition of 1 ml. of 0.1 N NaOH and 15 ml. of the appropriate buffer were added. 15 ml. of heptane were added and the mixture shaken for 15 minutes. The heptane phase was assayed for chloroquine as described in the method for 4-aminoquinolines (7) and the fraction of apparent drug extracted at each pH calculated.

Results of a comparison of a solution of pure chloroquine with the appar-

ent chloroquine extracted from plasma as described above are shown in Table IV. It is apparent that the heptane extract of plasma contains some material with solubility characteristics which differ from those of pure chloroquine by being water-soluble at the higher pH values. These data suggest that an alkaline wash of the heptane extract of the plasma will confer additional specificity on the analytical procedure.

TABLE IV

Distribution of Chloroquine and Apparent Chloroquine between Water and Heptane at Various pH Values

The apparent chloroquine was obtained by extraction, with heptane, of the plasma of a patient receiving the drug. The compound was then returned to dilute acid. Aliquots of this solution and of a chloroquine solution were adjusted to various pH values and shaken with equal volumes of heptane. The fraction of the compound extracted at the various pH values is expressed as the ratio of the amount of compound in the organic phase to total compound.

pH	Chloroquine, aqueous control (a)	Apparent chloroquine from plasma extract	
		Without alkali wash of solvent phase (b)	With alkali wash of solvent phase (c)
6	0	0	0
7	0	0.02	0
8	0.11	0.09	0.12
8.5	0.43	0.28	0.44
9	0.71	0.56	0.70
9.5	0.83	0.62	0.85
10	0.94	0 75	0.95
11	0.96	0.77	0.98
13 (0.1 N NaOH)	0.96	0.80	0.98
14 (1 N NaOH)	0.97	0.78	0.99

Column (a) pure chloroquine; column (b) apparent chloroquine from plasma extracted into heptane and returned to dilute acid; column (c) apparent chloroquine from plasma extracted into heptane and returned to dilute acid subsequent to removal of the drug metabolic derivatives by washing the heptane phase with 0.1 N NaOH.

The effect of the introduction of such a wash was studied as follows: A sample of plasma was extracted into heptane as described above and the heptane phase separated from the aqueous phase. 2 volumes of 0.1 N NaOH were added to the heptane and the mixture shaken 10 minutes. The alkali phase was separated from the heptane phase and set aside for subsequent analysis. This wash was repeated two additional times. The chloroquine in the washed heptane phase was then returned to 0.1 N H$_2$SO$_4$. Two 1 ml. aliquots of the solution were set aside for direct analysis. The remainder was shaken with a series of buffer solutions as described above.

The results in Table IV indicate that, as expected, the treatment with an alkali wash removes certain metabolic products of chloroquine from the heptane phase, leaving behind a substance with solubility characteristics similar to those of chloroquine.

The material in the alkaline washes was extracted into 2 volumes of ethylene dichloride and then returned to 6 ml. of 0.1 N HCl. 5 ml. aliquots of the acid phase were assayed for 4-aminoquinolines. The first alkaline wash was found to have removed 7 per cent, the second 4 per cent, and the third 2 per cent of the total 4-aminoquinolines. From this it may be concluded that with no washes of heptane extract of plasma the error due to the interference of metabolic products amounts to about 13 per cent, with one wash 6 per cent, and with two washes about 2 per cent. Similar results were obtained with plasmas from a number of patients to whom the drug had been administered.

It must be emphasized that an indication of specificity in the case of plasma does not necessarily indicate a similar specificity for urine and tissues. Many methods which have been found to be specific in the case of plasma require an additional step to remove interfering derived products present in urine and organ tissues. Furthermore, the specificity of a particular method applied to one species of animal may not carry over to another one.

SUMMARY

A scheme for the analysis of basic organic compounds in biological material, utilizing a number of simple general reactions, is described. This scheme provides for the isolation and concentration of compounds by extraction of the free base into an organic solvent, and thence into an aqueous phase. The organic solvent selected should exploit the finding that metabolic products of organic bases are commonly more water-soluble than the parent substance. Consequently the solvent of choice is one in which the compound is least soluble provided quantitative extraction is achieved. A large fraction of the metabolic products may thus be left behind in the initial extraction, the remainder removed by suitable washes. Extensive adsorption of organic bases on glass surfaces from non-polar solvents may be minimized by the addition of alcohol subsequent to the initial extraction.

A general technique to appraise specificity involves a comparison of the solubility characteristics of a pure compound with those of the apparent drug extracted from tissue. This examination yields information on solubility characteristics of interfering substances which may be used to modify the initial extraction procedure.

The final measurement is determined by the properties of the compound and involves fluorometry or microphotometry.

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THE ESTIMATION OF BASIC ORGANIC COMPOUNDS IN BIOLOGICAL MATERIAL

II. ESTIMATION OF FLUORESCENT COMPOUNDS*

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Many compounds fluoresce when they are irradiated with light, usually in the ultraviolet range. The extreme sensitivity as well as the simplicity of fluorometric assay makes it the method of choice in the design of analytical procedures. Fluorescence intensity depends upon such diverse environmental factors as intensity of irradiation, the nature of the solvent, temperature, pH, and the presence of quenching substances. These factors are controllable in an aqueous medium adjusted to the pH of maximal fluorescent intensity. The fluorescent properties of quinacrine, quinine, and many other organic bases have been utilized in the design of methods for their estimation in biological materials at concentrations as low as 5 γ per liter.

The organic base is isolated from the alkalized biological sample by extraction into a suitable organic solvent. Drug metabolic products not separated in the initial extraction may be selectively removed by alkaline washes of the solvent. The drug is then returned to dilute acid and the pH adjusted to that which will yield the maximal fluorescence. The concentration of the compound is estimated by measuring the fluorescence of the final solution. Larger concentrations of many fluorescent organic bases may be analyzed directly in the organic phase after the addition of trichloroacetic acid. This second procedure, though simpler, is usually less sensitive than the double extraction procedure.

Methods for the estimation of quinacrine are given below in detail to illustrate both general methods of analysis. Although adequate methods for the estimation of quinacrine have been described (1–3), the following methods are presented because they represent a logical application of the general scheme of analysis proposed in these papers. The procedures for

other fluorescent bases are the same in principle but may differ in the choice of solvent, achievement of specificity, and the pH and filter system that will yield the maximal fluorescence. These pertinent details for some fluores-

TABLE I

Data on Basic Organic Compounds That Can Be Estimated by Fluorescence; Double Extraction Method

Compound	Solvent	pH of maximal fluorescence	Filter system (Coleman filters)	Specificity (human plasma)
Quinacrine	Petroleum ether	9.5	B_4-PC_9	See foot-note 3
SN 5228. 2-Methoxy-6-chloro-9-(2-diethylaminoethylamino)acridine	" "	1 (0.1 N HCl)	"	Specific
SN 5341. 2-Methoxy-6-chloro-9-(4-diethylaminobutylamino)acridine	" "	9.5	"	Not examined
SN 9627. 2-Methoxy-6-chloro-9-(8-diethylaminooctylamino)acridine	Ethylene dichloride	9.5	"	Specific
SN 8439. 9-(4-Diethylamino-1-methylbutylamino)acridine	" "	9.5	"	Not examined
SN 8442. 3-Chloro-9-(4-diethylamino-1-methylbutylamino)acridine	" "	9.5	"	" "
Quinine	Benzene	1 (0.1 N H_2SO_4)	B_1-PC_1	Specific
Quinidine	"	1 (0.1 " ")	"	"
SN 6520. 1-Naphthol-2-(dimethylamino)methane	"	14 (1 N NaOH)	B_1S-PC_1	Not examined

cent basic compounds which have been tested as antimalarials in this laboratory are presented in Tables I and II.

Filter System—The choice of filter system depends on the wave-length of the exciting light, on the sensitivity requirements, and on whether inter-

fering fluorescence derived from the normal biological material is present. The filter combinations described below have been used in this laboratory but other combinations may be equally suitable.

A Corning Filter 587 (Coleman, B₁S) is used to isolate the 365 mμ line. The resulting fluorescent light is transmitted through a combination of Corning Filters 3389 and 4308 (Coleman, PC₁) The sensitivity of the measurement may be reduced by isolating the activating line with a filter partially covered by a neutral screen (Coleman, B₁). A further reduction

TABLE II

Data on Basic Organic Compounds That Can Be Estimated by Fluorescence; Single Extraction Method

Compound	Solvent`	Filter system	Specificity (human plasma)
Quinacrine	Heptane	B₂-PC₉	See foot-note 3
SN 5228. 2-Methoxy-6-chloro-9-(2 - diethylaminoethylamino)-acridine	"	"	Specific
SN 9627. 2-Methoxy-6-chloro-9-(8-diethylaminooctylamino)acridine	Ethylene di-chloride	"	"
SN 8341. 2-Methoxy-6-chloro-9-(4 - diethylaminobutylamino)-acridine	Heptane	"	"
Quinine	Benzene	B₁-PC₁	"
Quinidine	"	"	"
SN 9849. 6-Methoxy-2-phenyl-α-2 - (piperidyl)-4 - quinolinemeth-anol	Ethylene di-chloride	"	Not examined
SN 2157. 6-Methoxy-α(2-piperidyl)-4-quinolinemethanol	" "	"	" "
SN 3294. 4-(4-Diethylamino-1-ethylbutylamino) - 6 - methoxy-quinoline	" "	"	" "

of the sensitivity may be achieved by transmitting the fluorescent light through a Corning Filter 3385 (Coleman, PC₉).

A Corning Filter 5113 (Coleman, B₄) is used to isolate the 405 and the 436 mμ lines. The resulting fluorescent light is transmitted through a Corning No. 3385 (Coleman, PC₉) filter. When fluorescence measurements are taken in an organic solvent, the 436 mμ line is isolated with a combination of Corning Filters 5113 and 3389 (Coleman, B₂). This filter combination may result in a sensitivity less than with Filter 5113 alone, but it excludes blank fluorescence derived from the biological sample.

Procedure for Quinacrine

Reagents—

1. Standard solution of quinacrine, 100 mg. per liter.   127 mg. of the dihydrochloride dihydrate salt are dissolved in 1 liter of 0.1 N HCl.   This solution is stable when stored in the refrigerator.   Working standards are prepared daily by dilution with 0.1 N HCl.

2. 0.1 N NaOH.

3. Petroleum ether.   A technical grade of petroleum ether is purified by successive washing with 1 N NaOH, 1 N HCl, and water.

4. Isoamyl alcohol, reagent grade.

5. 0.1 N HCl.

6. 0.5 N NaOH.   1 ml. should be neutralized by 5 ml. of the above 0.1 N HCl.

7. Buffer reagent, pH 9.5.   5 volumes of 0.6 M boric acid in 0.6 M KCl are added to 3.2 volumes of 0.6 N NaOH.   When diluted as described in the procedure, the resulting pH should be 9.4 to 9.6.

Procedure, Double Extraction—Add 1 to 10 ml. of biological sample[1] (containing up to 1 γ of quinacrine) and an equal volume of 0.1 N NaOH to 30 ml. of petroleum ether in a 60 ml. glass-stoppered bottle.   Shake for 30 minutes on a shaking apparatus.   Allow the phases to separate, centrifuging the bottle if necessary.   Add 1 ml. of isoamyl alcohol, mixing with the petroleum ether so as not to disturb the aqueous phase.   Transfer 20 ml. of the petroleum ether phase to a 60 ml. glass-stoppered bottle or a glass-stoppered test-tube containing 6 ml. of 0.1 N HCl.[2]   Shake for 3 minutes and then centrifuge for 2 minutes at low speed.   Remove the supernatant organic phase by aspiration.   Transfer 5 ml. of the acid phase to a fluorometer tube containing 1 ml. of 0.5 N NaOH and 2 ml. of buffer reagent and mix thoroughly.   A "dummy" consisting of acid, alkali, and buffer is used for the blank setting of the fluorometer.   A reagent blank, in which water is substituted for plasma, is run through the same procedure.   This should read the same as the dummy with pure chemicals.

The standard is prepared by adding a known amount of the drug in 5 ml. of 0.1 N HCl to 1 ml. of 0.5 N NaOH and 2 ml. of buffer in a fluorometer tube.

The intensity of fluorescence of the samples is determined in the Coleman photofluorometer or other suitable instrument.   The filter system with the

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[1] Organ tissues and feces are emulsified in acid as described in Paper I of this series (4).   Quinacrine and other acridines are concentrated in the leucocytes of the blood, and therefore special precautions are required in the preparation of plasma for analysis (4).

[2] Halides which quench the fluorescence of quinine and quinidine should be avoided in their fluorescent measurement.   0.1 N H₂SO₄ can be used in the case of these compounds.

former consists of a 2 mm. No. 5113 Corning filter (Coleman, B₄) to isolate the activating energy, and Corning No. 3385 filter (Coleman, PC₉A) to transmit the resulting fluorescent light. Standards are run with each set of unknowns and the concentrations of the latter are estimated in relation to these standards. The standards give readings that are directly proportional to their concentration.

Procedure, Single Extraction—

Reagents—

1. 0.1 N NaOH.

2. Heptane. A technical grade of heptane is purified by successive washings with 1 N NaOH, 1 N HCl, and water. Petroleum ether is not used in this procedure because of its high fluorescent blank.

3. Isoamyl alcohol, reagent grade.

4. Trichloroacetic acid solution. 25 gm. of trichloroacetic acid are dissolved in 100 ml. of ethylene dichloride.

Add 1 to 5 ml. of biological sample (containing up to 1 γ of quinacrine) and an equal volume of 0.1 N NaOH to 15 ml. of heptane in a 60 ml. glass-stoppered bottle. Shake for 30 minutes on a shaking apparatus. Allow the phases to separate, centrifuging the bottle if necessary. Add 0.5 ml. of isoamyl alcohol, mixing with the heptane phase so as not to disturb the aqueous phase. Transfer about 10 ml. of the heptane phase to a fluorometer tube containing 1 ml. of the trichloroacetic acid solution. A dummy consisting of the heptane, isoamyl alcohol, and the trichloroacetic acid solution is used for the blank setting of the fluorometer. A reagent blank, with water in place of plasma, is run through the procedure. This should read the same as the dummy with pure chemicals.

The standard used in calibrating the sensitivity of the instrument is prepared by alkalizing an aqueous solution of quinacrine as above, extracting with heptane, and handling in the same manner as the biological sample. The computation of quinacrine concentration is by direct proportion.

The filter system consists of a Corning No. 5113 and 3389 combination filter (Coleman, B₂) to isolate the activating energy and Corning Filter 3385 (Coleman, PC₉) to transmit the resulting fluorescent light.

Results

It is shown in Table III that quinacrine added to plasma is recoverable with satisfactory precision, with the double extraction procedure, in amounts as low as 0.05 γ. Equally satisfactory results have been obtained with many other acridines, as well as with quinine and quinidine.

Table III also contains a summary of recoveries of known amounts of quinacrine from plasma by the single extraction procedure. The precision and sensitivity of the method are sufficient for amounts of quinacrine as low

TABLE III
Recovery of Quinacrine Added to Plasma

Quinacrine added	Quinacrine found	Recovery
	Double extraction method	
γ	*γ*	*per cent*
1.0	1.00	100
	1.00	100
	1.00	100
	1.04	104
0.5	0.50	100
	0.51	102
	0.50	100
	0.50	100
0.4	0.40	100
	0.38	95
	0.41	102
	0.41	102
0 3	0.29	97
	0.29	97
	0.29	97
	0.29	97
0 2	0.21	105
	0.19	95
	0.19	95
	0.21	105
0.1	0.11	110
	0.10	100
	0.11	110
	0.10	100
0.05	0.05	100
	0.05	100
	0.045	90
	0.055	110
	Single extraction method	
1.0	1.03	103
	0.99	99
	0.99	99
	1.00	100
0.5	0.52	104
	0.50	100
	0.48	96
	0.51	102
0.3	0.31	103
	0.315	105
	0.29	97
	0.31	103
0.2	0.21	105
	0.21	105
	0.21	105
	0.20	100

as 0.2 γ. Equally good results have been obtained for other fluorescent compounds.

Repeated analyses run on individual samples of plasma and urine stored in the refrigerator over a period of several weeks yield highly reproducible results, indicating that the drug in plasma and urine is stable under these conditions.

Specificity—There is a negligible amount of interfering fluorescent material in normal biological tissues. The extent of interference by metabolic products of the drug was studied by the technique described in Paper I of this series (4). This examination was made on petroleum ether extracts of

TABLE IV

Distribution of Quinacrine and Apparent Quinacrine between Water and Petroleum Ether at Various pH Values

The apparent quinacrine was obtained by extraction, with petroleum ether, of the plasma of a patient receiving the drug. It was then returned to dilute acid. Aliquots of this solution and of a quinacrine solution were adjusted to various pH values and shaken with equal volumes of petroleum ether. The fraction of the compounds extracted at various pH values is expressed as the ratio of the amount of compound in the organic phase to total compound.

pH	Quinacrine, aqueous control	Apparent quinacrine from plasma extract
7	0.05	0.05
8	0.50	0.55
9	0.90	0.90
10	0.95	0.98
14 (1 N NaOH)	1.00	0.97

plasma obtained from patients who received oral doses of the drug, which contained a total of 3 γ of apparent quinacrine. It is clear from the data in Table IV that petroleum ether extracts of plasma contain little fluorescent material which differs in solubility characteristics by this test from pure quinacrine.[3] Similar results were obtained with heptane extracts of plasma.

[3] Results obtained by the analysis of the plasma and urine from patients receiving quinacrine are the same as those obtained by the previous method with ethylene dichloride as the extracting solvent (2). In the latter method phenolic metabolic products of quinacrine were removed by means of an alkaline wash of the solvent. The use of a less polar solvent in the present method leaves the phenolic metabolic products behind in the initial extraction   Recent work indicates that a small amount of quinacrine metabolic product with solubility characteristics similar to the parent compound (Taggart, J. V., personal communication) is also measured in the present procedure. The presence of this product could not be detected by the technique used here in assaying specificity. This product, which was separated from quinacrine by a counter-current distribution technique, has ultraviolet and fluorescence characteristics similar to those of the parent compound. The extent of the interference in the plasma and urine of humans is of the order of 10 to 15 per cent.

SUMMARY

A simple general method is described which, with minor modification, is applicable to the determination of many organic soluble fluorescent bases. This method has been applied to the estimation of quinacrine in biological tissues for illustrative purposes. This procedure permits the determination of quinacrine in amounts as low as 0.05 γ. Pertinent information for the estimation by this method of certain other antimalarial drugs is also given.

Quinacrine is isolated from the biological material by an extraction of the free base with petroleum ether at an alkaline pH. The drug is then returned to acid and buffered to pH 9.5. The concentration of quinacrine is measured fluorometrically.

Higher concentrations of quinacrine are measured directly in a heptane extract after the addition of trichloroacetic acid.

A comparison of the solubility characteristics of quinacrine and the apparent quinacrine extracted from the plasma of patients who were receiving the drug indicates that the method has a high degree of specificity.

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THE ESTIMATION OF BASIC ORGANIC COMPOUNDS IN BIOLOGICAL MATERIAL

III. ESTIMATION BY CONVERSION TO FLUORESCENT COMPOUNDS*

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Non-fluorescent organic compounds may yield fluorophores on suitable treatment, the fluorescence of which may be used in the design of analytical methods. For example, the 7-chloro-4-aminoquinolines, an important group of antimalarial drugs, are changed to fluorophores when subjected to ultraviolet irradiation under suitable conditions. The resulting fluorophores are oxidized with loss of fluorescence with continued irradiation. This, however, may be prevented and the fluorophore stabilized by irradiating anaerobically or in the presence of cysteine.[1] Many of the 7-chloro-4-aminoquinolines appear to yield the same fluorophore, since the same relationship between molecular concentration and fluorescence intensity has been noted in all instances studied. The properties of the derived fluorophore have been utilized in the design of methods for the estimation of chloroquine (7-chloro-4-(1-methyl-4-diethylaminobutylamino)quinoline) and related compounds at concentrations as low as 10 γ per liter.

The compound is separated from the biological material by extraction into a suitable organic solvent at an alkaline pH. The solvent is freed of drug metabolic products, if necessary, with alkaline washes and the drug is then returned to dilute acid. The pH is adjusted to 9.5, cysteine is added, and the sample is irradiated with ultraviolet light. The concentration of the compound is estimated by measuring the fluorescence of the final solution.

The procedures for the various 4-aminoquinolines are the same in principle but may differ in choice of solvent and achievement of specificity. The procedure for chloroquine is given in detail below and the pertinent details for other 4-aminoquinolines which have been tested as antimalarials are presented in Table I.

[1] Masen, J. M., personal communication.

TABLE I

Data on Basic Organic Compounds That Can Be Estimated by Conversion to Fluorescent Compound

Compound	Solvent	Specificity for human plasma
Chloroquine. 7-Chloro-4-(1-methyl-4-diethylamino-butylamino)quinoline	Heptane	Specific after 2 washes of heptane extract with 2 volumes 0.1 N NaOH
SN 9584. 7-Chloro-4-(3-diethylaminopropylamino)-quinoline	" "	Specific after 1 wash of heptane extract with 2 volumes 0.1 N NaOH
SN 7135. 2-Methyl-7-chloro-4-(1-methyl-4-diethylamino-butylamino)quinoline	Ethylene dichloride	Specific
SN 7373. 7-Bromo-4-(1-methyl-4-diethylaminobutylamino)-quinoline	Heptane	Not examined
SN 13616. 7-Chloro-4-(4-ethylamino-1-methylbutylamino)-quinoline	" with 1.5% isoamyl alcohol	" "
SN 13425.* 7-Chloro-4-(1'-ethyl-4'-piperidylamino)quinoline	" "	Specific after 2 washes of heptane extract with 5% Na_2SO_4 in buffer, pH 11
SN 13588. 7-Chloro-4-(3-ethylaminopropylamino)quinoline	Heptane	Not examined
SN 10960.* 7-Chloro-4-(4-diethylamino-2-hydroxybutyl-amino)quinoline	"	" "
SN 14477.† · 7-Chloro-4-(4-diethylaminocyclohexylamino)-quinoline	"	Specific after 2 washes of heptane extract with 2 volumes 0 1 N NaOH
SN 8137.‡ 7-Chloro-4-(3-diethylamino-2-hydroxypropyl-amino)quinoline	"	Specific after 3 washes of heptane extract with 2 volumes 16% Na_2SO_4 in 1 N NaOH

* SN 13425 is slightly soluble in water. The metabolic products of the drug are removed from the heptane extract by means of washes with an alkaline solution of sodium sulfate. The removal of the parent drug is minimized by the use of this salt solution.

† Irradiated, and fluorescence read at pH 8.7.

‡ The extraction of SN 8137 from plasma is made with chloroform, since the extraction with heptane is incomplete for this tissue. However, the chloroform extract contains metabolic products of the drug which have fluorescent properties similar to it and which cannot be removed from this relatively polar solvent with alkaline washes. The SN 8137 is, therefore, returned to an aqueous phase and then to heptane. The metabolic products which enter the heptane are then removed by washes with an alkaline solution of sodium sulfate. A high salt concentration is essential in this step to minimize the loss because of the solubility of SN 8137 in the aqueous phase.

Procedure for Chloroquine

Reagents—

1. Standard solution of chloroquine, 100 mg. per liter. 161 mg. of the diphosphate salt are dissolved in 1 liter of 0.1 N HCl. This solution is stable when stored in the refrigerator. Working standards are prepared daily by dilution with 0.1 N HCl.

2. 0.1 N NaOH.

3. Heptane. A technical grade of heptane is purified by successive washings with 1 N NaOH, 1 N HCl, and water.

4. Absolute ethanol.

5. 0.1 N HCl.

6. 0.5 N NaOH. 1 ml. should be neutralized by 5 ml. of the above 0.1 N HCl.

7. Buffer reagent, pH 9.5. 5 volumes of 0.6 M boric acid in 0.6 M KCl are added to 3.2 volumes of 0.6 N NaOH. When diluted as described in the procedure and·after the addition of the cysteine reagent, the resulting pH should be 9.4 to 9.6. This pH should be checked by direct measurement.

8. 5 per cent cysteine reagent. 1 gm. of cysteine hydrochloride is dissolved in 20 ml. of water. This solution is neutralized by the addition of 0.8 ml. of 10 N NaOH. This reagent should be made fresh daily and neutralized just before use.

*Procedure—*Add 1 to 10 ml. of biological sample[2] (containing up to 1 γ of chloroquine) and an equal volume of 0.1 N NaOH to 30 ml. of heptane in a 60 ml. glass-stoppered bottle. Shake for 30 minutes on a shaking apparatus. Allow the phases to separate, centrifuging the bottle if necessary. Add 8 drops of ethanol and mix with the heptane phase so as not to disturb the aqueous phase. Transfer as much of the heptane phase as possible to a 125 ml. glass-stoppered bottle. Add about twice the volume of 0.1 N NaOH and shake for 5 minutes. After the two phases settle, add 8 drops of ethanol to the heptane phase and mix as before. Remove the aqueous phase by aspiration with a pipette inserted below the surface. Repeat the washing with 0.1 N NaOH. Then add 5 drops of ethanol and transfer 20 ml. of the heptane phase to a 60 ml. glass-stoppered bottle or a glass-stoppered centrifuge tube containing 6 ml. of 0.1 N HCl. Shake for 3 minutes and then centrifuge for 2 minutes at low speed. Remove the supernatant organic phase by aspiration. Transfer 5 ml. of the acid phase to a fluorometer tube containing 1 ml. of 0.5 N NaOH and 1.5 ml. of buffer

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[2] Organ tissues and feces are emulsified in acid as described in Paper I of this series. Chloroquine and other 4-aminoquinolines concentrate in the leucocytes of the blood and, therefore, special precautions are required in the preparation of plasma for analysis.

reagent. Add 0.5 ml. of cysteine reagent and mix thoroughly. A reagent blank, with water substituted for plasma, is run through the same procedure.

Standards—Standards are prepared by adding known amounts of the drug in 5 ml. of 0.1 N HCl to 1 ml. of 0.5 N NaOH and 1.5 ml. of buffer in fluorometer tubes. 0.5 ml. of cysteine reagent is added to each tube. A "dummy" consisting of acid, alkali, buffer, and cysteine reagent is used for the blank setting of the fluorometer; this should not change under the irradiation described below. After 30 minutes, to allow for reaction between cysteine and oxygen, all tubes are irradiated with ultraviolet light, as described below.

The intensity of fluorescence of the irradiated samples is determined in the Coleman photofluorometer or other suitable instrument. With the former, a Corning Filter 587 (Coleman, B₁S) is used to isolate the activating energy and a combination of Corning Filters 3389 and 4308 (Coleman, PC₁) to transmit the resulting fluorescent light. The sensitivity of the instrument is set by quinine, since the irradiated standards may slowly lose their fluorescence on frequent exposure to the ultraviolet light of the fluorometer. The sensitivity of the instrument should be set so that a solution of quinine in 0.1 N H_2SO_4 containing 0.0125 γ per ml. yields a reading of 50. 0.2 γ of irradiated drug in 8 ml. will then give a reading of about 40. Standards are run with each set of unknowns and the concentrations of the latter are estimated in relation to these standards. The standards are quite reproducible and give readings that are directly proportional to their concentration.

Irradiation of Samples—Irradiation of the 4-aminoquinolines is effected in a simply constructed irradiator. An H-4 mercury arc lamp with a No. 59G18 transformer (Hanovia) similar to those used in the Coleman photofluorometer is the light source. The samples are placed in a circular rack surrounding the lamp so that they are equidistant from the lamp. 3 hours are required to achieve maximal sensitivity. The time may be reduced to 1 hour by using a more intense source of ultraviolet light in the apparatus. This may be achieved by a DH-1 lamp with a No. 58G2 transformer (Hanovia). The time of irradiation must be worked out to meet the conditions in each laboratory, since too long an irradiation results in a gradual diminution of fluorescence. When the more intense light source is used, precautions must be taken to avoid the damaging effects of ultraviolet light on the eyes.

The temperatures of the solutions should be maintained below 35° during irradiation; otherwise degradation of the drug to less fluorescent or non-fluorescent products may occur within the time of irradiation. This cooling is effected in the irradiator by passing air around the lamp and tubes.

The use of the more intense light source may require additional cooling facilities, such as an electric fan.

A diagram of a suitable irradiator is shown in Fig. 1.
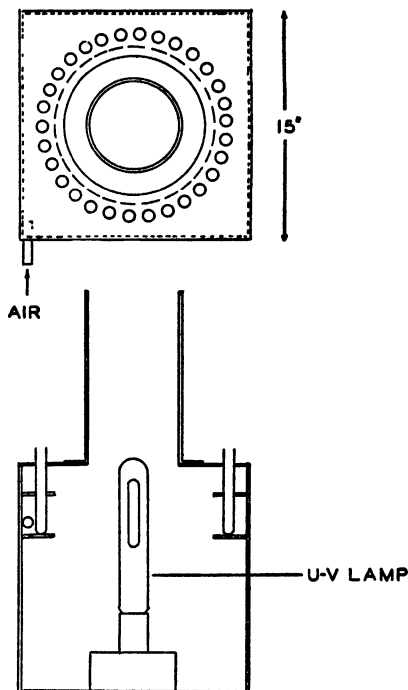
FIG. 1. Schematic diagram of the apparatus for the irradiation of solutions with ultraviolet light

Results

Recoveries of the drug from plasma are shown in Table II. The sensitivity and precision of the method are quite adequate for amounts as low as 0.2 γ. Equally good results have been obtained in the case of the other 4-aminoquinolines.

Repeated analyses run on individual samples of plasma and urine stored in the refrigerator over a period of a week yield highly reproducible results, indicating that the drug in plasma and urine is stable under these conditions.

Specificity—The absence of a detectable blank indicates that there is a negligible amount of material in normal biological tissues which reacts as chloroquine in the procedure described above. The possible interference of drug metabolic products of chloroquine has been examined by the tech-

nique described in Paper I of this series.[3] The results, which were described in that paper, indicate that metabolic products of chloroquine with fluorescent properties similar to the parent drug do occur in the plasma of patients receiving the drug. However, these are separated from the parent drug in the procedure described above. The interference due to the metabolic products, if no alkaline washes of the heptane extract are used, amounts to about 15 per cent. The alkali washes are therefore not essential for most routine determinations on plasma.

TABLE II

Recovery of Chloroquine Added to Plasma

Chloroquine added	Chloroquine found	Recovery	Chloroquine added	Chloroquine found	Recovery
γ	γ	*per cent*	γ	γ	*per cent*
0.2	0.21	105	0.4	0.37	92
	0.19	95		0.40	100
	0.18	90		0.36	90
	0.19	95		0 39	98
	0.20	100		0.41	102
	0.20	100		0.42	105
	0.17	85		0 40	100
	0.21	105		0 40	100
	0.21	105		0.37	92
	0.21	105		0 40	100
	0.20	100		0.41	102
	0.20	100	0.6	0 60	100
	0.20	100		0.60	100
	0.18	90		0 60	100
	0.17	85		0.60	100
	0 19	96		0 56	93
	0.21	105		0 56	93

SUMMARY

A simple method is described which, with minor modification, is applicable to the estimation of many 7-chloro-4-aminoquinolines in biological fluids and tissues. The method has been applied to the estimation of chloroquine for illustrative purposes. This procedure permits the estimation of chloroquine in amounts as low as 0.1 γ. Pertinent information for the estimation by this method of certain other antimalarial drugs is also given.

Chloroquine is isolated from the biological material by an extraction of the free base with heptane at an alkaline pH. Drug metabolic products

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[3] Brodie, B. B., Udenfriend, S., and Baer, J. E., *J. Biol. Chem.*, **168**, 299 (1947).

not separated in the initial extraction are selectively removed from the solvent by alkaline washes. The drug is then returned to acid solution and buffered to pH 9.5, cysteine is added, and the sample irradiated with a mercury vapor lamp. The concentration of fluorophore formed is measured fluorometrically.

A comparison of the solubility characteristics of chloroquine and the apparent chloroquine extracted from the plasma of patients who were receiving the drug indicates that the method is specific in that it includes, in the final measurement, no products due to the metabolism of chloroquine.

THE ESTIMATION OF BASIC ORGANIC COMPOUNDS
IN BIOLOGICAL MATERIAL

IV. ESTIMATION BY COUPLING WITH DIAZONIUM SALTS*

By BERNARD B. BRODIE, SIDNEY UDENFRIEND, AND
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Many aromatic amines in which the position para to the amino group is free react readily with diazonium salts to form compounds which absorb light in the visible range of the spectrum. This reaction may be used in the design of analytical procedures. It is subject to negligible interference from organic extractable substances normally occurring in biological material. Consequently, marked sensitivity may be achieved, if necessary, by coupling and making the final photometric measurement in a small volume. The coupling properties of various aromatic amines have been utilized in the design of methods for their estimation in biological material at concentrations as low as 25 γ per liter.

The aromatic amine is isolated from alkalized biological material by extraction into an organic solvent. The drug is then concentrated by returning it to a small volume of acid containing diazotized sulfanilic acid with which it couples. The resulting dye solution is assayed in a spectrophotometer or photoelectric colorimeter adapted to small volumes. Amines possessing a free phenol group are coupled with diazotized p-nitroaniline-o-sulfonic acid in neutral or slightly alkaline solution.

A procedure for the estimation of the antimalarial pamaquine (6-methoxy-8-(4-diethylamino-1-methylbutylamino)quinoline) in biological fluids is given as an illustration of the general method of analysis. The procedure for other amines may differ in choice of solvent and achievement of specificity.

A procedure for the estimation of another antimalarial, SN 5918 (4,4'-dihydroxy-3,3'-di(diethylaminomethyl)diphenyl ether) is given as an example of the estimation of an amine with a free phenol group.

Procedure for Pamaquine

General Considerations—The required sensitivity is obtained by concentrating the compound into a small volume and then utilizing a microphotometric technique for the final measurement. This is accomplished with the Coleman model 6 spectrophotometer by the use of a micro test-tube adapter which, without changing the length of the light path, reduces the cross-section of the solution through which the light must pass.[1] A ⅜ × 3 inch flat bottom test-tube is used to hold the solution. It is possible by this means to measure optical densities in volumes as small as 0.25 ml.[2]

A number of aromatic primary amines were diazotized and coupled with pamaquine. Of the diazotized amines examined, diazotized sulfanilic acid was found to be superior in respect to stability of the reagent, speed of coupling, and color intensity of the resulting dye. The coupling reaction is complete within 5 minutes. The resulting dye is stable for several hours. The absorption spectrum of the dye indicates that the wave-length of maximal light absorption is 480 mμ.

Reagents—

1. Standard solution of pamaquine, 100 mg. per liter. 163 mg. of the citrate salt are dissolved in 1 liter of 0.1 N H_2SO_4. This solution is stable when stored in the refrigerator. Working standards are prepared daily by dilution with 0.1 N H_2SO_4.

2. 0.1 N NaOH.

3. Petroleum ether. A technical grade of petroleum ether is purified by successive washings with 1 N NaOH, 1 N HCl, and water.

4. Isoamyl alcohol, reagent grade.

5. Coupling reagent. Solution A, 0.5 gm. of sulfanilic acid is added to 7.5 ml. of concentrated HCl and is then diluted to 500 ml. Solution B, 0.5 per cent sodium nitrite. These solutions are stored in the refrigerator.

Directions for Diazotization—The final coupling reagent is made up of 10 ml. of Solution A and 0.3 ml. of Solution B. After 10 minutes, to allow for complete diazotization of the sulfanilic·acid, the reagent is ready for use. The coupling reagent must be made fresh daily.

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[1] This tube adapter was patterned after a suggestion by Dr. Oliver Lowry of the New York City Department of Health. Adapters with tubes to fit may be obtained from Mr. Samuel Ash, 1115 East 105th Street, New York.

[2] Small concentrations of pamaquine may be measured with considerable accuracy if variations in the strength of the light source during a measurement are minimized. Increased stability of light source is obtained if the tungsten lamp is fed from storage batteries rather than from line current and transformer. The constancy of the light transmission should be verified after each measurement by checking the reading of the galvanometer needle after removal of the colorimeter tube. This air transmission in the case of the Coleman instrument is above the 100 per cent transmission mark

Procedure—Add 1 to 10 ml. of biological sample[3] (containing up to 10 γ of pamaquine) and an equal volume of 0.1 N NaOH to 30 ml. of petroleum ether in a 60 ml. glass-stoppered bottle. Shake for 30 minutes on a shaking apparatus. Allow the phases to separate, centrifuging the bottle if necessary. Add 2 ml. of isoamyl alcohol, mixing with the petroleum ether so as not to disturb the aqueous phase. Transfer 20 ml. of the petroleum ether phase to a tapered glass-stoppered centrifuge tube[4] containing 0.5 ml. of coupling reagent. Shake for 5 minutes and then centrifuge for 2 minutes at low speed. Remove the supernatant organic phase by aspiration.[5] Transfer 0.3 ml. of the aqueous phase to a micro colorimeter tube and determine the optical density of the dye solution at a wavelength of 480 mμ. Coupling reagent is used for the blank setting of the instrument. The same tube may be used for the blank setting and for all the determinations and standards. The tube is removed from the adapter after each reading and its contents removed by aspiration. The tube is then rinsed with acetone and dried by aspiration. It is advisable to make the measurements in a definite order, starting with the blanks, then the dilute solutions, and then the more concentrated samples.

Standards—Standards are prepared by taking 1 volume of standard solution and adding 9 volumes of coupling reagent. The optical density is read in the colorimeter after 5 minutes. Coupling reagent is used for the blank setting of the instrument. A permanent standard curve is constructed with optical density plotted against micrograms of pamaquine per ml. of solution. The optical densities were found to be proportional with all concentrations up to 4 γ per ml. An optical density of about 0.115 is obtained in the adapted Coleman model 6 when 1 γ of pamaquine is run through the procedure described above.

Results

Table I contains a summary of recoveries of known amounts of pamaquine added to plasma. The results indicate that the sensitivity and precision of the method are quite adequate for amounts as low as 0.25 γ. Equally satisfactory results have been obtained with other 8-aminoquinolines. Analyses run on plasma samples of pamaquine over a period of

[3] Organ tissues and feces are emulsified in acid as described in Paper I of this series.

[4] The glass-stoppered tubes used in this laboratory have a volume of about 40 ml. They are tapered at the bottom to a narrow cone to facilitate the manipulation of the small aqueous volume.

[5] Occasionally the aqueous solution of the dye will be turbid after the aspiration of the petroleum ether layer. Turbidity may be removed by shaking the solution with additional aliquots of petroleum ether and recentrifuging.

several weeks gave highly reproducible results. It may be concluded, therefore, that pamaquine in plasma is stable when stored in the refrigerator.

Specificity—The absence of detectable blanks indicates that there is a negligible amount of material in normal plasma and tissues which reacts as pamaquine in the analytical procedure described above. The degree of specificity is dependent only upon the extent to which the metabolic

TABLE I

Recovery of Pamaquine Added to Plasma

Pamaquine added	Pamaquine found	Recovery	Pamaquine added	Pamaquine found	Recovery
γ	γ	per cent	γ	γ	per cent
0.25	0.23	92	1.00	0.94	94
	0.255	102		0.94	94
	0.23	92		0.94	94
	0.25	100		0.96	96
	0.255	102		0.93	93
	0.23	92		0.94	94
	0.255	102		0.98	98
0.50	0.51	102		0 98	98
	0.50	100	2.00	1.90	95
	0.52	104		1.90	95
	0.51	102		2.02	101
	0.50	100		1.90	95
	0.46	92		1.90	95
	0.44	88		1.91	96
	0.485	97		1.90	95
	0.49	98		1.82	91
	0.48	96			
	0.47	94			
	0.48	96			
	0.55	110			
	0.47	94			
	0.49	98			

products are excluded. The examination of specificity has been made by the technique described in Paper I of this series.[6] These studies were made on petroleum ether extracts of plasmas which were obtained from patients who received oral doses of the drug and which contained 10 to 20 γ of apparent pamaquine. It is clear from the data shown in Table II that the petroleum ether extracts of plasma contain negligible amounts of material

[6] Brodie, B. B., Udenfriend, S., and Baer, J. E., *J. Biol. Chem.*, **168**, 299 (1947).

which react with the coupling reagent which differ in solubility characteristics from pure pamaquine.

<div align="center">TABLE II</div>

Distribution of Pamaquine and Apparent Pamaquine between Water and
Petroleum Ether at Various pH Values

The apparent pamaquine was obtained by extraction, with petroleum ether, of the plasma of a patient receiving the drug. The compound was then returned to dilute acid. Aliquots of this solution and of a pamaquine solution were adjusted to various pH values and shaken with equal volumes of petroleum ether. The fraction of the compounds extracted at various pH values is expressed as the ratio of the amount of compound in the organic phase to total compound.

pH	Plasma A		Plasma B	
	Aqueous control	Apparent pamaquine from plasma extract	Aqueous control	Apparent pamaquine from plasma extract
	(a)	(b)	(c)	(d)
5			0.15	0.20
6	0.39	0.41	0.48	0.52
7	0.78	0.77	0.86	0.81
8	0.93	0.90	1.01	0.96
9	0.93	0.94	0.97	1.03
10	0.96	0.98	0.97	1.03
11	0.97	0.99	1.01	1.03
13 (0.1 N NaOH)	0.99	1 02		
14 (1 N NaOH)	0 99	1.02	0.99	1.03

Columns (a) and (c), pure pamaquine; column (b), apparent pamaquine from 200 ml. of pooled plasma from ten patients, extracted into petroleum ether and returned to dilute acid; column (d), apparent pamaquine from 80 ml. of plasma from a single patient, extracted into petroleum ether and returned to dilute acid. For columns (a) and (b) measurements were taken at temperatures which differed from those for columns (c) and (d) by several degrees, which accounts for the difference in the two sets of measurements.

Procedure for SN 5918 (4,4'-Dihydroxy-3,3'-di(diethylaminomethyl)diphenyl Ether)

General Considerations—The sensitivity requirements for the estimation of this compound are not as critical as in the case of the 8-aminoquinolines because the plasma levels with therapeutic dosage are relatively high. It is not necessary, therefore, to make the final measurement in a small volume.

The large blank resulting from the instability of diazotized sulfanilic acid when it is used in neutral or alkaline solution precludes its use, but diazotized p-nitroaniline-o-sulfonic acid is satisfactory in this respect. The

absorption spectrum of the resulting dye indicates maximal absorption at 450 mμ. However, the reagent itself absorbs a considerable amount of light at this wave-length and it is necessary to make the readings at 520 mμ, at which the blank is negligible and the absorption of light by the dye is still sufficient for precise measurements.

Reagents—

1. Standard solution of SN 5918, 100 mg. per liter.   100 mg. of the free base are dissolved in 1 liter of 0.1 N HCl.   This solution is stable when stored in the refrigerator.   Working standards are prepared daily by dilution with 0.01 N HCl.

2. 1 N NaOH.

TABLE III

Recovery of SN 5918 (4,4'-Dihydroxy-3,3'-di(diethylaminomethyl)diphenyl Ether

SN 5918 added	SN 5918 found	Recovery
γ	γ	*per cent*
5	5.1	102
	5.1	102
	5.0	100
	5.1	102
10	11.1	111
	10.7	107
	10.7	107
15	15.4	103
	15.0	100
	14.5	97
	16.0	107

3. Benzene.   A technical grade of benzene is purified by successive washes with 1 N NaOH, 1 N HCl, and water.

4. 0.05 N HCl.

5. 0.5 N NaOH.

6. 0.5 M Na$_2$HPO$_4$.

7. Coupling reagent.   Solution A, 1.26 gm. of *p*-nitroaniline-*o*-sulfonic acid is added to 15.0 ml. of concentrated HCl and is then diluted to 1 liter. Solution B, 0.5 per cent sodium nitrite.   These solutions are stored in the refrigerator.

Directions for Diazotization—The final coupling reagent is made up of 10 ml. of Solution A and 0.3 ml. of Solution B.   After 10 minutes, to allow for complete diazotization of the *p*-nitroaniline-*o*-sulfonic acid, the reagent is ready for use.   This reagent, which must be made fresh daily, is diluted 1:5 for use in the procedure.

Procedure—Add 1 to 10 ml. of biological sample[3] (containing up to 50 γ

of SN 5918) and 1 ml. of 2.5 N NaOH to 30 ml. of benzene in a 60 ml. glass-stoppered bottle. Shake for 10 minutes on a shaking apparatus. Allow the phases to separate, centrifuging the bottle if necessary. Add 2 ml. of isoamyl alcohol and mix with the benzene phase so as not to disturb the aqueous phase. Transfer 20 ml. of the benzene phase to another bottle containing 6 ml. of 0.05 N HCl and shake for 5 minutes. Pour the contents of the bottle into a test-tube and centrifuge for 2 minutes at low speed. Remove the benzene phase by aspiration. Transfer exactly 5 ml. of the aqueous phase to a colorimeter tube. Adjust the pH by the addition of 0.5 ml. of 0.5 N NaOH and 0.5 ml. of 0.5 M Na_2HPO_4. Add 0.5 ml. of the diluted diazo reagent and then after 10 minutes add 0.5 ml. of 1 N NaOH. Determine the optical density at a wave-length of 520 mμ. Coupling reagent added to acid and buffered as above is used for the blank setting of the instrument.

Standards—These are prepared by taking 5 ml. of standard solution in 0.05 N HCl and adding buffer and coupling reagent as described above. A standard curve is constructed with optical density plotted against micrograms of SN 5918 per ml. of solution.

Results

The results detailed in Table III indicate that the sensitivity and the precision of the method are quite adequate for amounts as low as 5 γ. The degree to which metabolic products of the drug are excluded has not been examined.

SUMMARY

A simple general method is described which, with minor modification, is applicable to the determination of many aromatic amines in which the position para to the amino group is free. The method has been applied to the estimation of pamaquine in biological tissues for illustrative purposes. This procedure permits the determination of pamaquine in amounts as low as 0.25 γ.

Pamaquine is isolated from the biological material by an extraction of the free base with petroleum ether at an alkaline pH. The petroleum ether phase is shaken with a small volume of diazotized sulfanilic acid. Pamaquine reacts with the latter to form a water-soluble dye. The concentration of this dye is determined by the measurement of its optical density.

A comparison of the solubility characteristics of pamaquine and the apparent pamaquine extracted from the plasma of patients who were receiving the drug indicates that the method has a high degree of specificity in that it includes, in the final measurement, no products which are metabolic derivatives of pamaquine.

The method has also been applied to 4,4′-dihydroxy-3,3′-di(diethyl-aminomethyl)diphenyl ether, which is an aromatic amine containing a free phenol group. This compound is isolated from the biological material by an extraction of the free base with benzene at an alkaline pH. The drug is reextracted into acid and then coupled with diazotized *p*-nitroaniline-*o*-sulfonic acid at a slightly alkaline pH.

THE ESTIMATION OF BASIC ORGANIC COMPOUNDS IN BIOLOGICAL MATERIAL

V. ESTIMATION BY SALT FORMATION WITH METHYL ORANGE*

By BERNARD B BRODIE, SIDNEY UDENFRIEND, AND WESLEY DILL

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The methyl orange salts of most organic bases are highly soluble in certain organic solvents but relatively insoluble in water. Bases may, therefore, be assayed indirectly through the extraction of their methyl orange salt into an organic phase. The methyl orange which goes into the organic phase is measured photometrically. This is a general reaction and has been used in the design of analytical procedures for a number of alkaloids and synthetic basic organic compounds. The application of this reaction to the estimation of basic drugs in biological samples with ethylene dichloride as the solvent for the extraction has been described in a previous paper (1). The sensitivity of the reaction in this solvent is limited by some solubility of free methyl orange in ethylene dichloride and by complex formation of the dye with normally occurring organic bases extractable from the biological material. This limited the method to the analysis of drugs which are present in concentrations of about 0.5 mg. or more per liter of plasma. The use of the less polar solvent benzene, when possible, minimizes the above difficulties, since neither methyl orange nor the normally occurring biological substances which interfere are soluble in this solvent to an appreciable extent. Consequently, about a 10-fold increase in sensitivity may be achieved by returning the methyl orange from the benzene to a small volume of acid and assaying its concentration by microphotometry. This permits the estimation of cinchonine and many other compounds which are found in plasma at low concentrations after therapeutic dosage and which cannot be assayed by fluorescent or coupling methods. Concentrations in the order of 50 γ per liter of plasma may be estimated. An additional advantage of using the less polar solvent, benzene, is that separation of the drug from its metabolites is facilitated.

The organic base is separated from the biological sample by extraction

into benzene at an alkaline pH.   The benzene phase is shaken with methyl orange solution at pH 5 and the excess methyl orange is removed.   The methyl orange which dissolves in the solvent through salt formation with the organic base is returned to a small volume of acid and measured photometrically.

The development of a procedure for the estimation of cinchonine in biological material is given to illustrate the general method of analysis. The procedures for other bases are the same in principle, and the pertinent details for some basic compounds tested as antimalarials in this laboratory are presented in Table I.

TABLE I

Data on Basic Organic Compounds That Can Be Estimated by Methyl Orange Reaction

Compound	Specificity (human plasma)
Cinchonine	Specific
Cinchonidine	"
Paludrine. 1-(*p*-Chlorophenyl)-4-isopropylbiguanide	"
SN 4430. 1-(*p*-Chlorophenyl)-4-methyl-4-isopropyl-biguanide	Not examined
SN 8323. 2-(*p*-Chlorophenylguanidyl)-4-methyl-6-(diethylaminoethylamino)pyrimidine	" "
SN 8538. 2-Phenyl-4-α-piperidyl-1-quinolinemethanol	" "
SN 8233. 8-(4-Diethylamino-1-methylbutylamino)-5,6-dimethoxyquinoline	" "

Procedure for Cinchonine

Reagents—

1. Standard solution of cinchonine, 100 mg. per liter.   100 mg. of the free base are dissolved in 1 liter of 0.1 N H_2SO_4.   This solution is stable when stored in the refrigerator.   Working standards are prepared daily by dilution with 0.1 N H_2SO_4.

2. 2.5 N NaOH.

3. Benzene.   A technical grade of benzene is purified by successive washings with 1 N NaOH, 1 N HCl, and water.

4. Isoamyl alcohol.   A reagent grade of isoamyl alcohol is washed with 0.2 volume of 1 N HCl, followed by several washes with water.

5. Methyl orange solution.   Dissolve 90 mg. of the sodium salt of methyl orange in 100 ml. of 0.5 M boric acid solution by gentle heating, and cool the solution to room temperature.   Filter if necessary.   Wash the solution several times by shaking with an equal volume of ethylene dichloride. To test the solution dilute it 1:100 with 1 N HCl and measure the optical density in the Coleman model 6 spectrophotometer which has been adapted

to microphotometry (2). The optical density should read about 0.500 at 515 mμ.

6. 1 N HCl.

Procedure—Add 1 to 10 ml. of biological sample[1] (containing up to 5 γ of cinchonine) and 1 ml. of 2.5 N NaOH to 30 ml. of benzene in a 60 ml. glass-stoppered bottle and shake for 10 minutes, on a shaking apparatus. Allow the phases to separate, centrifuging the bottle if necessary. Add 0.5 ml. of isoamyl alcohol and mix with the benzene phase so as not to disturb the aqueous phase. Transfer as much of the benzene phase as possible to a glass-stoppered centrifuge tube containing 0.5 ml. of methyl orange reagent. Shake for 5 minutes and then centrifuge for 10 minutes at 2500 R.P.M. Transfer 20 ml. of the supernatant benzene phase to a tapered glass-stoppered centrifuge tube containing 0.5 ml. of 1 N HCl. Shake for 5 minutes and then centrifuge. Carefully remove the benzene phase by aspiration. Transfer at least 0.3 ml. of the aqueous phase to a micro colorimeter tube and determine the optical density of the methyl orange solution at a wave-length of 515 mμ, using the Coleman model 6 spectrophotometer adapted to micro spectrophotometry as described in the procedure for pamaquine (2).

A reagent blank in which water is substituted for plasma is run through the above procedure and is used for setting the instrument to zero optical density. This reagent blank should not give an optical density of more than 0.010 when 1 N HCl is used to set the instrument at zero optical density.

Standard Curve—Standards are prepared by taking 1 ml. of standard solution, adding 1 ml. of 2.5 N NaOH and 30 ml. of the benzene, and handling in the same manner as described for the biological sample. As noted above, a reagent blank is run through the same procedure and is used for setting the instrument to zero optical density. A permanent standard curve is constructed with optical density plotted against micrograms of drug. This is a highly reproducible linear relationship, so that standards need not be run together with each set of determinations. The optical densities were found to be proportional between 0.5 and 4 γ per ml. An optical density of 0.109 is obtained on the adapted Coleman model 6 spec-

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[1] Organ tissues and feces are emulsified in acid as described in Paper I of this series (3). There are substances in normal tissues which react with the methyl orange to give a considerable blank. To remove these the emulsified tissues are treated as follows· To 1 volume of emulsified tissue are added 5 volumes of 20 per cent metaphosphoric acid. This serves to precipitate the proteins and to remove most of the substances in tissue which react with the methyl orange to give a blank (4). An aliquot of the filtrate is made alkaline and extracted with benzene as described in the procedure.

trophotometer when 1 γ of cinchonine is run through the procedure as described above.

Results

A summary of the recoveries of known amounts of cinchonine added to plasma is shown in Table II.   It is evident from these results that the sensitivity and precision of the method are quite adequate for amounts of cinchonine as low as 0.5 γ.   Analyses run on single samples over a period

TABLE II

Recovery of Cinchonine Added to Plasma

Cinchonine added	Cinchonine found	Recovery
γ	γ	*per cent*
0.5	0.57	114
	0.51	102
	0.45	90
	0.50	100
	0.47	94
1	0.98	98
	1.00	100
	1.01	101
	1.06	106
	0.99	99
	0.94	94
2	2.0	100
	2.05	103
	1.95	98
	1.92	96
	1.90	95
	1.90	95
	1.85	93

of several weeks gave highly reproducible results, from which it may be concluded that the drug in plasma is stable when stored in the refrigerator.

Specificity—There is a small amount of material in normal biological tissues which reacts as cinchonine in the analytical procedure described above. This interference is negligible for most tissues when concentrations of the drug are high but for plasma in which it varies from 0 to 10 γ per liter it constitutes an error which limits the sensitivity of the method.

The possible interference by drug metabolic products has been tested. Cinchonine metabolizes in the body in the main to 2-hydroxycinchonine and to a derivative of the latter with an additional oxygen in the quinuclidine ring.[2]   Both of these compounds have been isolated from the urine

[2] Brodie, B. B., Baer, J. E., and Craig, L., unpublished work.

and have been shown to be present in plasma from persons receiving the drug. These compounds show a negligible interference in the method described above. The method, therefore, is highly specific.

Comment

Numerous organic bases form complexes with methyl orange which are quite soluble in benzene, so that the methyl orange enters the organic phase in amounts equivalent to the contained base. Other organic bases form complexes which distribute between water and the benzene phase, so that the amount of methyl orange which enters the organic phase is less than the equivalent base contained therein. It has been found in the latter instances that the standard curve varies somewhat from day to day and therefore standards must be run concurrently with each set of determinations. The solubility of the complex in the water phase may be decreased by using more concentrated methyl orange solutions. The methyl orange complexes are, as a rule, more soluble in the more polar solvent, ethylene dichloride. This solvent can often be used in those instances in which the complex is not soluble in benzene. However, it is preferable to use benzene when possible because of the low tissue and reagent blank and consequently the higher sensitivity.

SUMMARY

The sensitivity of the methyl orange reaction with organic bases has been increased for compounds which are extractable from biological material with benzene and whose complex with methyl orange is soluble in this solvent. For illustrative purposes, this revised method has been applied to the development of an analytical procedure for the estimation of cinchonine. This procedure permits the estimation of cinchonine in amounts as low as 0.5 γ. Pertinent information for the estimation by this method of certain other antimalarial drugs is also given.

Cinchonine is isolated from the biological material by an extraction of the free base with benzene at an alkaline pH. The benzene is shaken with methyl orange solution. The methyl orange which dissolves in the solvent through salt formation with cinchonine is returned to a small volume of acid and its concentration is estimated by determining the optical density.

The method as applied to cinchonine has a high degree of specificity.

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THE ESTIMATION OF BASIC ORGANIC COMPOUNDS IN BIOLOGICAL MATERIAL

VI. ESTIMATION BY ULTRAVIOLET SPECTROPHOTOMETRY*

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Many basic organic compounds exhibit characteristic absorption maxima in the ultraviolet region. Ultraviolet spectrophotometry lacks the sensitivity of the methods of assay previously described in this series of papers, but it may be useful as a final means of assay for compounds not readily amenable to analysis by other methods. It can also be used as an aid in developing procedures which may eventually involve other principles of assay (1). Ultraviolet spectrophotometry has been applied to the quantitative estimation of organic bases in plasma[1] at concentrations in the order of 1 mg. per liter.

The organic base is isolated from alkalized plasma by extraction into a suitable organic solvent. Normally occurring interfering substances are removed from the plasma extract by means of an aqueous alkaline wash. The drug is then returned to 0.1 N H_2SO_4 and the concentration of the compound is estimated spectrophotometrically.

A procedure for the estimation of quinine in plasma is given to illustrate this method of analysis. Quinine in 0.1 N H_2SO_4 possesses an absorption maximum at 250 mμ, and measurements are therefore made at this wavelength. The procedures for other bases are the same in principle, differing in the choice of solvent and the wave-length of maximal light absorption.

General Considerations

Instrument—The instrument used was the Beckman quartz photoelectric spectrophotometer. A recent simple adaptation of this apparatus

[1] The measurement of drug concentration in organ tissues has not been attempted as yet in this laboratory.

permits the use of small volumes without changing the length of the light path through the solution (2). It is possible that the sensitivity of the method described below may be markedly increased by using this adaptation.

Solvents—Heptane and ethylene dichloride have been used as the extracting solvents. The purification of these solvents involves the removal of those substances which are extractable by acid and which interfere in the final measurement. The solvents are routinely purified by shaking successively with 1 N NaOH and 1 N HCl, followed by two washes with water. Technical grades of solvent have been found satisfactory.

Blanks—The limiting factor in the sensitivity of the absorption measurements is the reagent blank. The blank due to the reagents is minimized by purifying the solvents as described above. The glass-stoppered tubes used in the final acid extraction, the pipettes used in transferring the acid solution to the cuvette, and the cuvettes are cleaned with chromic acid. The optical density of the reagent blank should not be greater than 0.005 when measured between the wave-lengths 230 to 300 mμ. At higher wave-lengths the blank is negligible.

Ethylene dichloride and, to a lesser degree, heptane, extract normally occurring organic bases from plasma which also absorbs light between 230 and 300 mμ. These substances may be selectively removed from the solvent extract by an aqueous alkaline wash at about pH 9. For this purpose, an aqueous solution of sodium borate is used.

Procedure for Quinine

Reagents—

1. Standard solution of quinine, 100 mg. per liter. 100 mg. of the free base are dissolved in 1 liter of 0.1 N H_2SO_4.

2. 2.5 N NaOH.

3. Ethylene dichloride. A technical grade of ethylene dichloride is purified as described above.

4. 0.02 N sodium borate.

5. 0.1 N H_2SO_4.

Procedure—Add 1 to 4 ml. of plasma and 1 ml. of 2.5 N NaOH to 15 ml. of ethylene dichloride in a 60 ml. glass-stoppered bottle and shake for 10 minutes on a shaking apparatus. Transfer the contents to a test-tube and centrifuge at 3000 R.P.M. for 5 minutes. Remove the aqueous phase by aspiration. Transfer as much of the solvent phase as possible to a 60 ml. glass-stoppered bottle. Add about 3 volumes of the sodium borate solution and shake for 5 minutes. Remove the aqueous phase by aspiration. Repeat the washing. Then transfer 10 ml. of the solvent phase to a 60 ml. glass-stoppered bottle or to a glass-stoppered test-tube containing 4

ml. of 0.1 N H_2SO_4. Shake for 3 minutes and then centrifuge at low speed. Transfer about 3 ml. of the aqueous phase to a quartz cuvette and read the optical density in a spectrophotometer, with the instrument set at the wave-length 250 mμ. A reagent blank, with water substituted for plasma, is run through the same procedure. This should not have an optical density greater than 0.005.

Working standards of the drug are prepared by dilution of the stock standard with 0.1 N H_2SO_4. The samples give readings on the spectrophotometer which are directly proportional to their concentration. A "dummy" consisting of 0.1 N H_2SO_4 is used for the blank setting of the

TABLE I

Recovery of Quinine Added to Plasma

Ouinine added	Quinine found	Recovery
γ	γ	per cent
5	4.95	99
	5.05	101
	4.94	99
	5.03	101
10	10.1	101
	10.1	101
	9.97	100
	9.88	99
20	19.8	99
	20.0	100
	19.5	98
	19.7	99

spectrophotometer. A concentration of quinine of 5 γ per ml. should read about 0.430 in terms of optical density.

Results

The data in Table I indicate that the sensitivity and precision of the method are adequate for amounts of quinine as low as 5 γ. Normal plasma extracts contain negligible amounts of material which interfere in the procedure described above. The extent of interference by metabolic products of the drug has not been assayed.

SUMMARY

A simple spectrophotometric method is described which is applicable to the estimation of many organic soluble bases which absorb light in the ultra-violet region. This method has been applied for illustrative purposes

to the estimation of quinine in plasma. This procedure permits the determination of quinine in amounts in the order of 5 γ.

Quinine is isolated from the plasma by an extraction of the free base with ethylene dichloride at an alkaline pH. Normally occurring substances which interfere in the absorption measurement are selectively removed by an aqueous wash at pH 9. The drug is then returned to acid and the concentration measured spectrophotometrically at 250 mμ.

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THE PLASMA AMINO ACID AND AMINO NITROGEN CONCENTRATION DURING NORMAL PREGNANCY, LABOR, AND EARLY PUERPERIUM*

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The α-amino nitrogen in plasma during normal pregnancy, labor, and the early puerperium has been determined by the gasometric ninhydrin-carbon dioxide method and is reported in this communication. For comparison, the amino nitrogen has also been determined simultaneously by the photometric method with sodium β-naphthoquinone-4-sulfonate.

EXPERIMENTAL

Subjects—Blood specimens were obtained by venipuncture from women who were pregnant, in labor, and in the early puerperium. All these women were normal clinically. Blood specimens were also obtained from women who were admitted to the gynecological service for minor surgical procedures. These specimens were used to determine the normal non-pregnant values.

The blood specimens taken during pregnancy were obtained as the women registered at or attended the antenatal clinic. These patients had eaten varying amounts of different foods for breakfast, and the bloods were taken at varying times following the meal. However, no indication has been obtained that ingestion of breakfast has any marked effect upon the values obtained. This is probably due to the fact that the breakfasts consumed were generally light and contained only small amounts of protein. All other blood specimens were withdrawn from patients before breakfast.

Methods—The free amino acid nitrogen in picric acid filtrates of plasma was determined by the gasometric ninhydrin-carbon dioxide method of Hamilton and Van Slyke (1). None of these data are corrected for urea.

The amino nitrogen in Wu filtrates of plasma (2) was determined by the Folin reaction as described by Frame, Russell, and Wilhelmi (3) and by Russell (4). The transmission of the final colored solutions was determined with an Evelyn photometer (5) with Filter 520.[1]

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[1] Frame, Russell, and Wilhelmi (3) recommend Filter 490. When this work was started we did not have this filter. We have subsequently determined that for all practical purposes it makes very little difference whether one uses Filter 520 or 490 if average values are compared as in this paper. It is true, of course, as pointed out by the original authors, that the resolving power is greater when Filter 490 is used.

Urea was determined directly on the plasma by the Conway micro diffusion method (6) as modified by Steinitz (7).

Results

The plasma amino acid nitrogen during pregnancy and during labor averages about 3.2 mg. per cent (Table I). This is approximately 25 per cent less than the values observed in normal non-pregnant women. The

TABLE I

Plasma α-Amino Acid Nitrogen during Pregnancy*

Lunar month of pregnancy	No. of specimens	Mean	Range	σ
		mg per cent	*mg. per cent*	
3	11	3.3	2.9–3.7	0.30
4	19	3.2	2.5–3.7	0.26
5	14	3.3	2.6–4.0	0.39
6	18	3.1	2.1–4.0	0 40
7, 8	10	3.2	2.8–3.7	0 28
9, 10	13	3.3	3 0–3 7	0.26
	85	3.2	2 1–4 0	

* About 90 per cent of these determinations were carried out in duplicate.

TABLE II

Plasma α-Amino Acid Nitrogen, Amino Nitrogen, and Urea of Normal Non-Pregnant, Pregnant, and Parturient Women

	No. of subjects	Ninhydrin method		Photometric method		$\frac{M_1 - M_2}{\sigma_D}$	Urea, average
		Average	σ	Average	σ		
		mg per cent		*mg. per cent*			*mg. per cent*
Normal	15	4.2 (3 8–4 8)	0.29	4.5 (3.3–6 2)	0 69	1 54	12.0
Pregnant	39	3.2 (2.1–4 0)	0.39	3 5 (2 3–4 7)	0 56	2 78	8.7
Labor	18	3 0 (2 4–3.7)	0 28	3 9 (2 8–6.2)	0 81	4 31	9.4
Postpartum	25	4.3 (3 5–5.0)	0 33	5 4 (4 1–6.8)	0.71	7.05	12.6

amino acid nitrogen has reached this level by the 9th week of pregnancy. Two plasmas have been obtained from women in their 6th week of pregnancy. The amino nitrogen was decreased at this time in these two. No significant change in amino acid nitrogen level occurs during pregnancy. No change takes place during labor. There are no indications in the determinations performed upon plasma from eighteen patients who had been in active labor for varying lengths of time (from 1 to 53 hours, with

the majority ranging from 1 to 16 hours of labor) that there would be any particular change in the value as a function of the time in labor or the character of the labor.

The free amino acid nitrogen values return to normal shortly after delivery. The present data are not sufficient to delimit the exact time. However, normal values may be observed on the 1st and 2nd postpartum days.

The values obtained by the photometric method for amino nitrogen are generally higher than those obtained by the more specific ninhydrin method. Such a result was expected. In the normal and pregnant series the photometric method gave values some 7 and 10 per cent higher, respectively, than those obtained by the ninhydrin method (Table II). This difference is of the same order of magnitude as that which Hamilton and Van Slyke (1) observed between the values obtained with the ninhydrin method compared with those by the Van Slyke nitrous acid method (8). The results obtained by the photometric method during labor and during the early puerperium are 30 and 26 per cent higher, respectively, than the corresponding values by the ninhydrin method. This result was not expected. At the present writing it cannot be explained.

DISCUSSION

The values obtained here for the plasma amino acid nitrogen in the normal non-pregnant individual are of the same order of magnitude as has been previously reported by others (1, 9–12). In so far as we know, this is the first report of the determination of the plasma amino acids by the gasometric ninhydrin-carbon dioxide method during pregnancy, labor, and the early puerperium.

The reason for this decrease in the plasma amino nitrogen is not clear. It may be in part due to the increase in blood volume which occurs during pregnancy (13). But if this were the only factor involved, a gradual decrease in the amino acid nitrogen as a function of the time elapsed since conception might be expected. Such a relationship does not seem to exist. No reciprocal relationship exists between the amino acid nitrogen and the urea nitrogen, since both values are decreased in pregnancy (14). But the urea seems to decrease as a function of the length of pregnancy, whereas the amino nitrogen remains constantly low. Nor does there seem to be any correlation between the plasma amino-nitrogen and the plasma uric acid. According to our results the concentration of this latter compound in the plasma is definitely decreased in early pregnancy and possibly tends to increase somewhat toward term.[2]

[2] Bonsnes, R. W., unpublished data.

The value obtained in this study for plasma amino nitrogen by the β-naphthoquinone-4-sulfonate reaction is definitely lower than the 4.5 mg. per cent reported by Plass and Mathew (15) by the original Folin procedure. Therefore, it may not be comparable because of the modifications in procedure and improvements in instrumentation which have occurred since then. It is interesting to note, however, that the plasma value reported by Plass and Mathew for the pregnant woman is significantly lower than that reported by both Folin and Berglund (16) and by Wu (2) when the data of these investigators are evaluated statistically.[3] Since all three investigators used essentially the same method at about the same time, their data should be comparable.

The significantly higher values obtained by the photometric method as compared with the gasometric ninhydrin-carbon dioxide method during labor and during the early puerperium cannot be explained. The increase may be due, in part, to the increase in uric acid which supposedly occurs during that time (17–19). If uric acid alone were responsible, a correlation should exist between the uric acid value and the amino nitrogen value. But when such data are plotted, no apparent correlation appears. However, the data have not been subjected to any rigorous mathematical analysis. Further data along these lines are being collected. In view of these results, amino nitrogen as determined by the β-naphthoquinone-4-sulfonate method may not justifiably be translated into amino acid nitrogen unless both the β-naphthoquinone and the ninhydrin methods give approximately similar results under similar experimental conditions.

Increases in amino nitrogen during labor and the puerperium have been reported previously. Scontrino (20) reported an increase in the amino nitrogen of trichloroacetic acid filtrates of plasma as determined by the Van Slyke nitrous acid method (8) and Doneddu (21) has observed an increase in the amino nitrogen of whole blood filtrates as determined by the Folin method during this period. Both these investigators found the increase to occur during the last weeks of pregnancy. Scontrino also found the amino nitrogen to be higher in early pregnancy than in the

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[3] These calculations have been carried out by us in the usual manner, using the data which appear in the original papers. Both Wu (2) and Plass and Mathew (15) give the actual data, and so no assumptions need be made. However, Folin and Berglund (16) give an average value and the range of values, from which it is possible to calculate the maximum and minimum variance possible (but not necessarily permissible). A median possible variance was then calculated from these two figures. This calculated variance agreed well with one calculated from an assumed reconstruction of the data. Any errors involved in making these assumptions will not change the order of magnitude of the result sufficiently to alter the conclusion that the average value is significantly different from the one reported by Plass.

normal non-pregnant individual. Doneddu observed normal values during the early puerperium. Contrariwise, Morse (22) interpreted his limited data as indicating no change in the amino nitrogen of trichloroacetic acid filtrates of both whole blood and plasma during labor. Our own data do not indicate any rise in the amino nitrogen in the plasma until the time of labor, and even then some of the values lie within the range observed during pregnancy.

The lower amino acid and amino nitrogen content of the plasma during pregnancy reported here necessitates a reevaluation of the amino nitrogen levels in whole blood during pregnancy. Previously the amino nitrogen of whole blood has been reported unchanged during pregnancy (*i.e.*, essentially the same as in a non-pregnant individual) as determined by both the colorimetric (15, 21, 23) and the gasometric nitrous acid methods (22). These observations have been confirmed by us many times before the plasma amino acid nitrogen had been determined. Since the whole blood amino nitrogen is within normal limits and the plasma amino acid nitrogen and the amino nitrogen are decreased and the hematocrit is also decreased, it follows that the amino nitrogen (but not necessarily the amino acid nitrogen) of the cells during pregnancy is increased. A preliminary survey indicates that the compounds present in the cells which react with β-naphthoquinone-4-sulfonate do not all react with ninhydrin. The cells seem to have a normal content (*i.e.*, normal non-pregnant value) of compounds which react with ninhydrin and a much higher content of compounds which react with β-naphthoquinone-4-sulfonate. When sufficient of these data have been collected, they will be presented in a separate paper.

SUMMARY

The α-amino acid nitrogen and the amino nitrogen in the plasma during pregnancy, labor, and the puerperium have been determined by the gasometric ninhydrin-carbon dioxide and by the photometric β-naphthoquinone-4-sulfonate methods, respectively. The α-amino acid nitrogen was found to average about 3.2 mg. per cent during pregnancy and labor and to return to a normal of about 4.3 mg. per cent during the early puerperium. The amino nitrogen averaged 3.5 mg. per cent during pregnancy, 3.9 mg. per cent during labor, and 5.4 mg. per cent during the early puerperium. These last two values are significantly different from those obtained by the gasometric ninhydrin procedure.

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THE COMPOSITION OF CARDIOLIPIN*

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The isolation of the phospholipide cardiolipin from beef heart was first reported in 1941 (1) and some of its chemical properties have been discussed in subsequent reports in connection with improvements in methods of preparation (2–4). The present paper deals with a study of the products of alkaline hydrolysis. The data presented have been collected from numerous experiments in which samples of several lots of cardiolipin were used.

Cardiolipin is easily saponified with potassium hydroxide in absolute alcohol at room temperature, when the greater part of the phosphorus-containing portion of the molecule is obtained as a precipitate of alcohol-insoluble potassium salts. This water-soluble, phosphorus-containing fraction is a complex ester which breaks down to glycerol and glycerophosphoric acid on acid hydrolysis. The soap solution yields fatty acids amounting to about 73 per cent of the weight of cardiolipin, identified as oleic and linoleic acids. It does not appear that any significant proportion of the molecule is left unaccounted for, and one can therefore formulate the general structure of the compound on the basis of the data presented here.

EXPERIMENTAL

Hydrolysis of Cardiolipin

To a solution containing 1.03 gm. of sodium cardiolipin in 150 ml. of absolute alcohol were added 1.5 gm. of carbonate-free KOH, dissolved in absolute alcohol. The solution became cloudy within a minute of mixing and the precipitate then increased rapidly, flocculating when the mixture was shaken. The mixture was allowed to stand at room temperature with occasional shaking for about 2 hours, at which time the supernatant was clear. It had previously been found that longer hydrolysis did not, increase the yield of the precipitate. This initial precipitate was designated Fraction K-1 (see flow sheet, below).

The mixture was centrifuged and the precipitate thoroughly washed with several portions of absolute alcohol. To the alkaline solution and washings were added a further 5 gm. of KOH dissolved in a little water, making the final concentration of KOH about 3 per cent. The solution was allowed to stand overnight at room temperature to insure complete saponification.

Fatty Acids—The alcoholic soap solution was concentrated *in vacuo*, diluted with water, acidified with H_2SO_4, and extracted with petroleum ether. Further extraction of the acid aqueous solution with ether removed only 4 mg. of brownish oily material. The nearly colorless petroleum ether extract was washed with water until the washings were free of sulfate; it was then dried overnight on anhydrous Na_2SO_4, filtered, concentrated, and finally transferred to a weighed flask and evaporated to dryness. The residue was dried to constant weight in a desiccator from which air was displaced with nitrogen before evacuation to about 2 mm. The fatty acids weighed 0.751 gm. or 73.3 per cent of the weight of cardiolipin hydrolyzed. The average of four such determinations gave a value of 73.0 per cent fatty acids.

Acid Aqueous Fraction—After extraction with ether, the acid aqueous solution was neutralized to litmus with KOH and concentrated to dryness. The salt mass was repeatedly extracted with warm methyl alcohol; the solution so obtained was evaporated to dryness and the residue was taken up in absolute methyl alcohol and filtered from a trace of K_2SO_4. The slightly brownish solution was designated Fraction K-2. It was made up to 25 ml. and aliquots were analyzed for phosphorus; found, 4.45 mg. of P or 10.3 per cent of that originally present.

Fraction K-1—The precipitate formed in the original saponification mixture was a dense white powder, readily soluble in water and extremely hygroscopic. It was also soluble in absolute methyl alcohol. Aliquots of a methyl alcohol solution were taken for analysis; found, 37.97 mg. of P or 88.2 per cent of the original. Hence 98.5 per cent of the phosphorus of cardiolipin was recovered in the form of methyl alcohol-soluble K salts by this method of hydrolysis.

Fatty Acids

The fatty acids isolated from the petroleum ether extract formed a nearly colorless viscous oil. The molecular weight by titration was 279.2. Iodine numbers were determined by Yasuda's modification of the Rosenmund-Kuhnhenn method (5); the mean of the values found for eight separately prepared lots of fatty acid was 163, the values ranging from 159 to 167. The high iodine number made it appear unlikely that any

saturated acids were present; nevertheless a sample of 1.4 gm. was examined by the lead salt-alcohol method (6). No saturated acid fraction could be detected.

Hydrogenation—A sample of the freshly isolated acids weighing 0.841 gm. and having an iodine number of 163 was dissolved in 95 per cent alcohol and hydrogenated in the presence of platinum oxide catalyst (7). The reduced acids obtained after filtration and evaporation of the alcohol formed a crystalline mass weighing 0.82 gm., which was recrystallized once from 10 ml. of acetone. A trace of material insoluble in the boiling acetone was removed by filtration. This substance, which has not been identified, weighed 16 mg.; it was insoluble in ether or petroleum ether, slightly soluble in chloroform and in hot ethyl alcohol. A little more was probably lost in filtering off the catalyst.

The recrystallized fatty acid separated in glistening plates, weighing 0.644 gm. The molecular weight by titration was 283. The melting point was 68.5–69° and there was no depression when the sample was mixed with known stearic acid. A second crop of crystals obtained from the concentrated mother liquor weighed 0.11 gm. and melted at 67.5–68°.

The high yield of stearic acid, amounting to 92 per cent of the reduced acid recovered, practically limited the possible components of the original unsaturated mixture to oleic, linoleic, and linolenic acids. The high iodine number proved that either linoleic or linolenic acid must be present.

In a preliminary attempt to detect linolenic acid, samples of the fatty acid mixture were brominated in chloroform and in ether solutions at −5°. Most of the product was an oil, only a very small yield of crystalline tetrabromostearic acid being obtained. No ether-insoluble bromide could be detected.

Another sample, 1.30 gm., was brominated in petroleum ether at −5° to −10° as recommended by Brown and Frankel (8). The yield of solid tetrabromostearic acid was 1.13 gm.; for recrystallization, this was dissolved in 10 ml. of ether and the solution was centrifuged to remove an insoluble residue, which weighed 15 mg. The clear ether solution was mixed with 20 ml. of petroleum ether and allowed to stand overnight at 3–6°, when the tetrabromostearic acid separated in glistening rosettes, m.p. 113–113.5°. According to Brown and Frankel's formula (8, 9), the yield of tetrabromostearic acid would correspond to a tetrabromide number of 86.9 or 84.5 per cent of linoleic acid. A mixture of 84 per cent linoleic acid with 16 per cent oleic acid would have an iodine number of 167.

The chief fatty acids of cardiolipin are therefore linoleic and oleic in the approximate ratio 5:1. The presence of small amounts of other acids is indicated by the occurrence of a trace of ether-insoluble bromide and the acetone-insoluble contaminant in the reduced acid fraction.

Water-Soluble Components

In preliminary experiments on Fraction K-1 it was found that most of the material could be converted to Ba salts freely soluble in 50 per cent alcohol. Only very small amounts of glycerophosphate could be detected. The acid corresponding to the soluble Ba salt could not be prepared intact: hydrolysis evidently took place readily in acid solutions, and the recovered mixtures always contained considerable amounts of glycerophosphoric acid. After Fraction K-1 had been boiled with HCl or H_2SO_4, two products, glycerol and glycerophosphoric acid, could be recovered. Glycerol was identified as the tribenzoate. A little free phosphoric acid was also formed. In Fraction K-2, obtained after acidification of the soap solution, practically all the phosphorus was present as glycerophosphoric acid, with traces of free phosphoric acid.

TABLE I

Recovery of Cleavage Products of Cardiolipin

Found on analysis		Radical in original molecule	
	per cent		*per cent*
Fatty acids	73 3	$C_{17}H_{31}CO$	68 59
Phosphorus	4 08*	PO_2	8.29
Glycerol	17 1	$C_3H_5O_2$	16 54
Sodium	2 72†	Na	2 72
Total	.		96.14

* Found by analysis of intact cardiolipin, 4 18 per cent (see Table IV)
† Previously reported (2)

These findings suggested the presence of a complex glyceryl-glycerophosphate ester. Further experiments were designed to show whether such a structure could account for the whole of the water-soluble portion of the cardiolipin molecule and whether the complex ester could be isolated in an analytically pure state.

A sample of sodium cardiolipin weighing 2.02 gm. was saponified and Fractions K-1 and K-2 were separated as described above. Aliquots of these two solutions were analyzed for phosphorus and glycerol.[1] Fraction K-1 contained 76.0 mg. of P and 317 mg. of glycerol; Fraction K-2, 5.6 mg. of P and 29 mg. of glycerol. In Table I the data are recalculated to allow for the water added during hydrolysis. It is evident that the recovery of material is essentially complete.

[1] Phosphorus was gravimetrically determined as phosphomolybdate, essentially by the method of Elek (10). Glycerol was determined by the method of Blix (11)·

Acid Hydrolysis of Fraction K-1—75 ml. of an aqueous solution of Fraction K-1 containing 0.76 mg. of P per ml. were mixed with 15 ml. of concentrated HCl to give a final acid concentration of about 2 N and refluxed in an all-glass apparatus for 3 hours. The hydrolysate was evaporated to dryness and HCl removed by repeatedly adding small portions of water and reevaporating to dryness. The residue was finally dissolved in water and the solution was transferred to a 50 ml. centrifuge tube and neutralized to phenolphthalein with saturated Ba(OH)$_2$. A small flocculent precipitate formed. This was collected by centrifugation and washed three times with water. It was then redissolved in a little dilute HCl and the solution was filtered from a trace of insoluble matter and again neutralized with Ba(OH)$_2$. The re-formed Ba salt was again thoroughly washed with water and finally with alcohol, followed by ether, and dried *in vacuo;* weight, 0.0764 gm. ˙

All supernatants and washings from the purification of the insoluble fraction were added to the main lot of water-soluble Ba salt. The solution was concentrated to 20 ml. and mixed with 20 ml. of alcohol, yielding a coarsely flocculent precipitate of barium glycerophosphate. This was redissolved in water, centrifuged to remove a further trace of the insoluble fraction, and again precipitated with an equal volume of alcohol. The precipitate was washed with alcohol and ether and dried *in vacuo;* weight, 0.4130 gm. A second crop was obtained by concentrating the 50 per cent alcoholic mother liquors to 10 ml. and precipitating with 20 ml. of alcohol; this weighed 0.0457 gm. and was added to the main lot. For analysis, a sample of the pooled Ba glycerophosphate fraction was dissolved in water, freed from a trace of the insoluble fraction, reprecipitated with an equal volume of alcohol, and dried to constant weight at 140° *in vacuo.*

$C_7H_8O_4PBa.$ Calculated. Ba 44.54, C 11.68, P 10.05, glycerol 29.85

Found. " 44.95, " 11.77, " 10.19, " 29.53

The 50 per cent alcoholic mother liquors from purification of the glycerophosphate were evaporated to dryness, the residue was extracted with acetone, and aliquots of the clarified acetone solution were analyzed by the Blix method to determine free glycerol.

The results are summarized in Table II. The two major products, glycerol and glycerophosphoric acid, account for most of the material.

Fractionation of Fraction K-1—The major component of this fraction was isolated in the form of its Ba salt. ˙

Fraction K-1 from the saponification of 1.87 gm. of cardiolipin was dissolved in absolute methyl alcohol and the solution made up to 50 ml.; it contained 1.412 mg. of P per ml., or 90.3 per cent of the total phosphorus. To 48.0 ml. of this solution, containing 67.8 mg. of P, were added 1.5 ml. of 20

per cent aqueous BaCl$_2$. A heavy flocculent precipitate formed. The mixture was allowed to stand 2 hours in the ice box at 3–6° and the precipitate was then collected by centrifugation, washed several times with methyl alcohol, dissolved in water, and the solution made up to 50 ml. Analysis showed 1.176 mg. of P per ml., representing 78.3 per cent of the total phosphorus and 86.7 per cent of the phosphorus in Fraction K-1. The material thus precipitated as the Ba salt was designated Fraction A.

The supernatant and washings from Fraction A were concentrated *in vacuo* almost to dryness and dissolved in water. The clear aqueous solution was warmed with a slight excess of K$_2$SO$_4$ and the BaSO$_4$ precipitate washed several times with hot water. The resulting aqueous solution of K salts was evaporated to dryness *in vacuo* and the residue was extracted

TABLE II

Products of Acid Hydrolysis of Fraction K-1

Fraction	Phosphorus		Glycerol	
	mg.	*per cent of total*	*mg.*	*per cent of total*
Crude Fraction K-1	56.9	100	238	100
Ba glycerophosphate	46.5	81.7	138	58
Insoluble Ba salt* .	8.17	14.4	6.4	2.7
Organic P . .	3.57	6.3		
Phosphate P .. .	4.60	8.1		
Free glycerol . . .			84	35.3
Total recovered	54.67	96.1	228.4	96.0

* Dissolved in dilute HCl and aliquots taken for analysis. Inorganic P was determined by precipitating the sample directly with the Pregl molybdate reagent. In a control test, a glycerophosphate sample allowed to stand overnight with the reagent did not split off any phosphate. The difference between the inorganic P and the total found after fusion gave the organically bound P.

with several portions of warm methyl alcohol. The insoluble portion consisted of K$_2$SO$_4$ and was discarded. The methyl alcohol solution was evaporated to dryness and the residue treated with absolute ethyl alcohol, in which most of it was soluble. The ethyl alcohol solution, Fraction B, contained 6.43 mg. of P or 9.5 per cent of the total in Fraction K-1; the small insoluble portion, which was soluble in methyl alcohol and appeared to be the K salt corresponding to Fraction A, contained 1.98 mg. of P or only 2.9 per cent of the total.

It is not clear as yet whether the alcohol-soluble K salt here isolated could have been present as such in the original precipitate of Fraction K-1. Since no reagents other than neutral salts were used and since the separation of Fractions A and B took place merely by precipitation of the Ba salt of Fraction A with BaCl$_2$, it would seem that hydrolysis of an original

compound of Fractions A and B is excluded. Fraction B might have been held in the original precipitate by adsorption on the insoluble K salt of Fraction A. Further study of Fraction K-1 by other methods would be necessary to determine this point.

The alcoholic solution of the K salt of Fraction B gave a fairly heavy flocculent precipitate on the addition of alcoholic $CaCl_2$. The Ca salt so prepared was readily soluble in methyl alcohol. Attempts to purify it by reprecipitation with absolute ethyl alcohol from aqueous or methyl alcoholic solution were unsuccessful, as its solubility in the mixed solvents was too great. One sample was partly purified by first washing thoroughly with ethyl alcohol, then dissolving the precipitate in methyl alcohol and filtering from a little insoluble matter. The methyl alcohol-soluble salt was dried for analysis; found, P 13.61, C 21.24, Ca 7.28. This fraction was presumably a mixture.

Purification of Ba Salt of Fraction A—Several lots of the Ba salt, totaling 3 gm., were pooled for purification. The salt was dissolved in 25 ml. of H_2O and the slightly cloudy solution was mixed with 25 ml. of alcohol. A very small oily precipitate formed, which was redissolved in 5 ml. of H_2O and mixed with 5 ml. of alcohol; it then yielded a flocculent precipitate of Ba glycerophosphate. After two more reprecipitations from 50 per cent alcohol the glycerophosphate weighed 0.31 gm. or about 10 per cent of the total.

The pooled 50 per cent alcohol solutions were concentrated *in vacuo* to about 25 ml. and poured into 250 ml. of absolute methyl alcohol. The flocculent precipitate was collected after refrigerating overnight. The cycle of solution in water, precipitation with an equal volume of alcohol, and recovery by precipitation into methyl alcohol, was repeated four times more in order to remove further traces of glycerophosphate and water-insoluble Ba salts. The purified salt, dried by washing with alcohol and ether, weighed 1.25 gm.; it was a dense hygroscopic white powder readily soluble in water or 50 per cent ethyl alcohol. A second crop of 1.1 gm. was recovered by concentrating the dilute methyl alcohol mother liquors and once more precipitating with absolute methyl alcohol.

For analysis, the purified salt was dried to constant weight *in vacuo* at 140°. The results are presented in Table III, together with the calculated values for compounds containing two, three, and four glycerophosphate radicals. The presumable structure of the compound is discussed below; its composition agrees well with that calculated for a compound made up of 3 glycerophosphoric acid molecules and 1 additional molecule of .glycerol (Table III, column (b)).

Thus, the water-soluble fraction of hydrolyzed cardiolipin consisted largely of a single substance, Fraction A, which was easily separated as the Ba salt. Second in amount was the rather ill defined Fraction B,

differing from Fraction A in the solubility of its K, Ca, and Ba salts in ethyl and methyl alcohols, but quite probably not a single compound. The other three fractions, glycerophosphoric acid, the acid giving a water-insoluble Ba salt, and phosphoric acid, accounted for only a small proportion of the total.

TABLE III

Analysis of Ba Salt, Fraction A

	Found		Calculated*		
	Top fraction	Second crop	(a)	(b)	(c)
Ba. ...	27.05	27.45	25.66	27.21	28.06
C†	19.06	19 12	20.19	19.04	18.41
P..	12.25	12 04	11 57	12.28	12 66
Glycerol	46.71	46.51	51.58	48.63	47.03

* Values calculated in per cent for the following formulas: (a) $C_9H_{20}O_{13}P_2Ba$ (2 glycerophosphoric acid + 1 glycerol); (b) $C_{12}H_{26}O_{18}P_3Ba_{\frac{3}{2}}$ (3 glycerophosphoric acid + 1 glycerol); (c) $C_{15}H_{32}O_{23}P_4Ba_2$ (4 glycerophosphoric acid + 1 glycerol).

† Carbon was determined by the manometric method of Van Slyke and Folch (12). For the carbon determinations reported in this paper the author is indebted to Dr. W. R. Thompson.

The results of the hydrolysis are presented in condensed graphic form in the accompanying chart.

Hydrolysis of Cardiolipin

Cardiolipin + KOH

Supernatant
Acidify, extract with
petroleum ether

Ppt. Fraction K-1
CH_3OH solution + $BaCl_2$

Fatty acids

Fraction K-2
10% of P

Soluble
Fraction B
10–12% of P

Ppt.
Ba salt
Fraction A
78% of P

Glycerophosphoric acid,
free glycerol

Mixture (?) glyceryl-
glycerophosphate
esters

Glyceryl-trigly-
cerophosphate
(Formula II)

DISCUSSION

Satisfactory criteria of the purity of an amorphous substance such as cardiolipin are difficult to establish. Repeated purification over the Cd

salt has not been found to result in significant changes in chemical or serologic properties, so far as these have been studied. It was therefore felt that cardiolipin purified in the routine manner (4) was sufficiently pure for chemical study. However, small amounts of unidentified contaminants were undoubtedly still present in the samples used for these experiments. The presence of traces of unidentified acids in the fatty acid mixture might be accounted for by such impurities. Further study of methods for more rigid purification of cardiolipin is desirable; meanwhile, the present results are sufficient to establish the general structure of the compound.

In drawing conclusions from the analytical data, it is to be remembered that the Blix method is not specific for glycerol, and a portion of the "glycerol" found by analysis might conceivably be some other alcohol giving a volatile iodide. However, no evidence for the presence of such another alcohol was found. Glycerol was also qualitatively identified as the tribenzoate. It is therefore a reasonable assumption that the skeleton of the molecule is a polyester of glycerol and glycerophosphoric acid.

In earlier studies it was established that cardiolipin functions as a monobasic acid; thus the ratio Na:P in the neutral Na salt was approximately 1:1 (2), and titration of the free acid showed a single acid valence for each phosphorus atom (3). The glyceryl-glycerophosphate structure in which each phosphoric acid group is doubly esterified is in accord with these findings. The simplest glyceryl-glycerophosphoric acid would be:

$$\begin{array}{c} O \\ \parallel \\ CH_2OH \cdot CHOH \cdot CH_2O \cdot P \cdot OCH_2 \cdot CHOH \cdot CH_2OH \\ \mid \\ OH \end{array}$$

(I)

Larger molecules could obviously be built up by similar linkage with more glycerophosphoric acid. Assuming such a structure, the composition of the purified Ba salt isolated from Fraction K-1 agrees best with that calculated from the following formula of the free acid.

$$\begin{array}{c} O \qquad\qquad\qquad O \qquad\qquad\qquad O \\ \parallel \qquad\qquad\qquad \parallel \qquad\qquad\qquad \parallel \\ CH_2OH \cdot CHOH \cdot CH_2O \cdot P \cdot OCH_2 \cdot CHOH \cdot CH_2O \cdot P \cdot OCH_2 \cdot CHOH \cdot CH_2O \cdot P{-}OH \\ \mid \qquad\qquad\qquad\quad \mid \qquad\qquad\qquad\quad O \\ OH \qquad\qquad\qquad OH \qquad\qquad\qquad CH_2 \\ \qquad\qquad\qquad\qquad\qquad\qquad\qquad\qquad CHOH \\ \qquad\qquad\qquad\qquad\qquad\qquad\qquad\qquad CH_2OH \end{array}$$

(II)

The ester linkages are arbitrarily written in the α position. There is no evidence on this point as yet: either α or β linkages or both might be present, and numerous isomers are obviously possible. It is also clear that incomplete hydrolysis of this ester could give a complex mixture of products, depending on which linkages were broken. It is interesting to note that the second ester linkage in the polyester is quite easily hydrolyzed, in contrast to the great stability of glycerophosphoric acid itself.

Two interpretations of the results obtained from the study of Fraction K-1 are possible: (a) Fraction A, having Formula II above, represents the intact skeleton of the cardiolipin molecule, while the other fractions are products of partial hydrolysis; or (b) Fraction A is only a part of a

TABLE IV

Composition of Sodium Cardiolipin

	Found	Calculated*		
		(a)	(b)	(c)
Carbon, %	64.98	65.66	65.13	64.60
Phosphorus, %	4.18	4.23	4.20	4.17
Sodium, %	2.72	3.14	3.12	3.09
Fatty acids, % .. .	73 0	76.63	76.01	75.40
Glycerol, %	17.5	16.78	16 64	16.58
Iodine No.	120–125	127 2	126 2	125.1
Mol. wt. per P atom	740–748	732	738	744

* (a) Calculated for the formula $C_{120}H_{208}O_{24}P_3Na_3$ (Formula II), with eleven double bonds (five linoleyl radicals and one oleyl), mol. wt 2195; (b) formula (a) + 1 molecule of H_2O; (c) formula (a) + 2 molecules of H_2O.

larger complex originally present, which breaks down into Fractions A and B during saponification. Assumption (a) seems somewhat more plausible, especially since as much as 78 per cent of the total phosphorus can be isolated in the form of Fraction A. If (a) is correct, cardiolipin has the structure given in Formula II, with the hydroxyl groups esterified by linoleic and oleic acids. The formula contains six available hydroxyl groups, or two for every phosphorus atom, and it may be recalled that linoleic and oleic acids were found in a ratio of 5:1. Thus it is at least a plausible assumption that cardiolipin represents a single compound of constant fatty acid composition, in contrast to the case of lecithin and the cephalins, which are mixtures of compounds of different fatty acids.

Table IV presents a comparison of the analytical data obtained with the values calculated on the assumption that the structure of cardiolipin corresponds to Formula II. It will be seen that the agreement is reason-

ably close, particularly if the further assumption is made that the molecule may be hydrated.

The identification of a glyceryl-glycerophosphate ester as a component of natural products has apparently not been previously reported. On account of the lability of such esters to acid hydrolysis, they could not be isolated after cleavage of a phospholipide by the common method of boiling with hydrochloric or sulfuric acids. Recent studies (13, 14) have demonstrated the occurrence of a variety of complex phospholipides in plant and animal sources, and other examples will doubtless be found. It would be interesting to know whether the glyceryl-glycerophosphate structure occurs in other phospholipides than cardiolipin.

Among previously known phospholipides the nearest analogy to cardiolipin is the group of phosphatidic acids discovered by Chibnall and Channon in plants (15, 16) and formulated by them as diglyceride phosphoric acids. Like cardiolipin, the phosphatidic acids occur naturally in the form of salts, and it may be noted that the solubility of their salts as described by Chibnall and Channon is similar in general to that of the corresponding salts of cardiolipin.

SUMMARY

The products of alkaline hydrolysis of cardiolipin were identified as linoleic acid, oleic acid, and a polyester of glycerophosphoric acid and glycerol. A tentative formula is presented. Cardiolipin is best classified as a complex phosphatidic acid.

Further work on the structure of cardiolipin is in progress.

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THE ISOLATION OF THE 6-PYRIDONE OF N¹-METHYLNICOTINAMIDE FROM URINE

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The 6-pyridone of N¹-methylnicotinamide was first identified as the product of the oxidation *in vitro* of N¹-methylnicotinamide by the quinine-oxidizing enzyme of rabbit liver. It was subsequently isolated from human urine after nicotinamide administration (1). The isolation, identification, and properties of this new derivative of nicotinamide are described in this paper.

Enzymic Formation of the Pyridone—The product of N¹-methylnicotinamide oxidation was made by incubating N¹-methylnicotinamide aerobically with purified quinine-oxidizing enzyme prepared as previously described (2). An excess of enzyme (50 ml. containing 70 cinchonidine units per ml.) was used to oxidize 100 mg. of N¹-methylnicotinamide chloride at pH 8.5 and 37°. The reaction was taken to completion by following the oxygen uptake of a pilot run. The characteristic absorption curve of the product, which can be observed in a metaphosphoric acid filtrate of the reaction mixture, was used to follow the compound through the isolation procedure. After the solution was deproteinized with metaphosphoric acid, it was neutralized, saturated with sodium carbonate, and then continuously extracted with isobutyl alcohol. The alcohol extract was dried with anhydrous calcium sulfate, evaporated, and the residue taken up in boiling acetone. Upon slow evaporation of the acetone solution, 30 mg. of reddish needles in rosettes separated. After three recrystallizations from acetone, nearly white crystals with a constant melting point of 212–214° were obtained.

Analysis—$C_7H_8O_2N_2$. Calculated. C 55.24, H 5.31, N 18.42
                        Found. " 55.15, " 5 31, " 18.45

The compound is readily soluble in water and alcohol, and nearly insoluble in less polar solvents. Both aqueous and alcoholic solutions have the same absorption curve, which changes little, if at all, throughout the pH range and which is identical with that seen in the deproteinized enzyme solutions (Fig. 1).

Isolation of Pyridone from Urine—The method for isolating this compound from urine is based on the behavior of the enzymically prepared pyridone subjected to various fractionating procedures. The urine was

collected with acetic acid as preservative and kept refrigerated until used. It was decolorized by precipitation with 3 gm. of basic lead acetate per 100 ml. at pH 10, filtered, adjusted to pH 2, and refiltered. Lloyd's reagent, 10 gm. per 100 ml., was then added and filtered off after 15 minutes. The Lloyd's reagent was washed with 0.01 N HCl, and then twice eluted with absolute ethyl alcohol. The alcohol eluate was evaporated, and the residue dissolved in a small volume of water.

The aqueous solution was saturated with sodium carbonate and extracted twice with equal volumes of isobutanol. The isobutanol solution was dried with calcium sulfate and then evaporated. The residue was
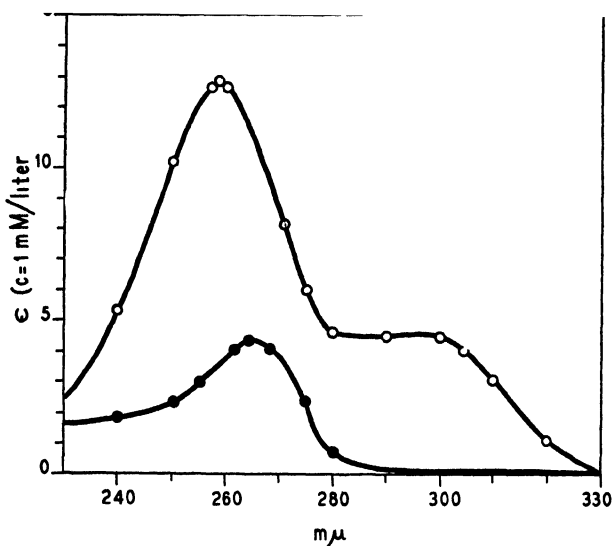
FIG. 1. Absorption curves of 6-pyridone of N^1-methylnicotinamide (O) and of N^1-methylnicotinamide chloride (●) in water at pH 7.

repeatedly extracted with hot acetone. Crystals were obtained from the acetone solution after reducing its volume and allowing it to stand overnight in the cold. Additional crops were obtained by further evaporation. Upon recrystallization from acetone, or more conveniently from water, a low melting impurity is removed, and the resulting compound melted at 211–214°. The mixed melting point with the enzymically prepared pyridone was unchanged, and the absorption curves of the compounds from both sources are identical.

N^1-Methylnicotinamide subjected to this isolation procedure was lost at the adsorption step, and no pyridone was formed in the procedure.

Identification of the Pyridone—The quinine-oxidizing enzyme specifically

oxidizes, in the α position, certain heterocyclic rings that contain an active α-hydrogen. The compound prepared by the action of the enzyme on N^1-methylnicotinamide and also isolated from urine must consequently be one of the two α-pyridones of N^1-methylnicotinamide. This formulation is consistent with the elementary analysis.

It was possible to identify the compound isolated from both sources as the 6-pyridone of N^1-methylnicotinamide by hydrolyzing it to the corresponding acid, since both the 2- and the 6-pyridones of N-methylnicotinic acid are known and have widely different melting points. Hydrolysis of 100.2 mg. of the pyridone from urine was carried out by refluxing in sodium methylate under nitrogen until the calculated amount of amide ammonia was freed (70 hours). The solution was acidified and evaporated to dryness and the product taken up in absolute ethyl alcohol. After distilling the alcohol, the brown residue was dissolved in 6 ml. of boiling water. About 60 mg. of white needles crystallized out on cooling. These melted at 239–240° after two recrystallizations from water.

Analysis—$C_7H_7O_3N$. Calculated. C 54.90, H 4.61, N 9.15
Found " 54.80, " 4.37, " 9 36

The 6-pyridone of N-methylnicotinic acid, synthesized from coumalic acid and methylamine (3), melted at 240–241° (m.p. 238–240° (4)), and the mixed melting point with the acid prepared by hydrolysis was 237–241°. The melting point of the other isomer, the 2-pyridone of N-methylnicotinic acid, is given as 183° (5). The acid from the hydrolysis was also converted to the methyl ester. This melted at 137°, in agreement with the melting point recorded for the ester of the 6-isomer (6). The pyridone isolated from the enzyme reaction and from urine is, therefore, the 6 derivative (1-methyl-3-carboxylamide-6-pyridone).

Relation of the Pyridone to Nicotinamide Assays—By the previous methods used to determine the derivatives of nicotinamide apparently little or none of this compound has been assayed. The pyridone cannot replace nicotinic acid in the bioassay with *Lactobacillus arabinosus*, nor coenzyme I for growth of *Hemophilus parainfluenzae*. It fluoresces only slightly under the conditions used for N^1-methylnicotinamide analysis (7), and is eliminated by either the charcoal or the Decalso adsorption steps usually employed in this procedure. In addition, the pyridone forms no color in the general reaction for N-methylnicotinamide compounds developed by Sarett (8), nor in the cyanogen bromide reaction (9). New analytical procedures applicable to this compound will be published later.

SUMMARY

1. A new metabolite of nicotinamide, detected by study of the enzyme oxidizing N^1-methylnicotinamide and subsequently isolated from urine,

has been identified as the 6-pyridone of N^1-methylnicotinamide (1-methyl-3-carboxylamide-6-pyridone).

2. Approximately 10 per cent of administered nicotinamide was isolated from human urine as the pyridone

3. This compound has apparently not been estimated by previous methods for the determination of nicotinamide derivatives in urine.

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THE ACTION OF TYROSINASE ON CHYMOTRYPSIN, TRYPSIN, AND PEPSIN

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The action of the enzyme tyrosinase on proteins has recently been made the subject of a study by Sizer (1). He has claimed that among a variety of proteins tested those which contained tyrosine could be oxidized by tyrosinase either directly or after pretreatment with trypsin. The proteins most susceptible to the action of tyrosinase were said to be the proteolytic enzymes chymotrypsin, trypsin, and pepsin. Furthermore it was stated that the proteolytic activities of these enzymes were not decreased after the oxidation by tyrosinase of a certain part of the tyrosine residues, and the inference was drawn that the intactness of at least a part of the tyrosine residues was not essential for the action of the enzymes. This question has been reinvestigated in this laboratory as it seemed to us that for several reasons the experimental conditions used by Sizer did not permit an unequivocal interpretation.

Methods and Results

We have in our experiments tried to keep the experimental conditions as close as possible to those used by Sizer.

The crystalline proteolytic enzymes were all prepared in this laboratory (2-4). Stock solutions of the enzymes were made up in dilute hydrochloric acid at pH 3 to 4 and at a concentration of 50 mg. per ml. calculated on dry substance. The chymotrypsin and trypsin preparations contained about 50 per cent magnesium sulfate. The protein concentration was calculated from the Kjeldahl nitrogen.

The tyrosinase preparation used was prepared from common mushrooms[1] and had an activity of 500 Miller and Dawson catecholase units (5) and 16 Adams and Nelson p-cresolase units per ml. (6).

The oxygen uptake was followed in the Warburg apparatus at 37.0° and at a shaking rate of 120 complete oscillations per minute. All experiments were performed in the following way, with one exception stated below. To the reaction flask which had a volume of about 5 ml. were added 1.6 ml. of M/15 phosphate buffer, pH 7.3, 0.2 ml. of the neutralized

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[1] Kindly supplied by Professor John M. Nelson of the Department of Chemistry, Columbia University, New York.

proteolytic enzyme solution, and 1 drop of toluene. Into the side arm was introduced 0.4 ml. of the tyrosinase solution, together with 1 drop of toluene. It was found necessary to let the vessels shake in the constant temperature bath for 2 to 3 hours in order to attain gas equilibrium. After that time the experiment was started by mixing the solutions. When, at the end of the experiment, the oxygen uptake had ceased, tyrosine was added in order to test the activity of the tyrosinase. In all the experiments the tyrosinase was found still to be active. Controls were also run, differing only in that boiled tyrosinase solution was substituted for the active tyrosinase.

In order to find the equivalency of tyrosine to oxygen in the oxidation by tyrosinase, runs have been made with various amounts of tyrosine oxidized by tyrosinase to completion. On the average 1 mg. of tyrosine took up 205 microliters of oxygen. An equivalency of 3 atoms of oxygen per 1 mole of tyrosine would correspond to 186 microliters of oxygen.

Non-protein tyrosine and tryptophane were determined in the trichloroacetic acid filtrate by the method of Folin and Ciocalteau with Anson's modifications (7). These determinations were made both on the stock enzyme preparation prior to the incubation and on the control with boiled tyrosinase at the end of the experiment. This figure cannot be compared directly with the tyrosine equivalent of the oxygen absorbed, since the former represents both tyrosine and tryptophane.

The reactions were also followed by measurements of the proteolytic activity. In addition to the measurements of protease activity after incubation with boiled or active tyrosinase, the activity was also determined before incubation to determine the effect of the experimental conditions on the proteases. No difference in activity was ever observed between the samples incubated with active tyrosinase and those incubated with the inactive preparation. Chymotrypsin activity was determined by the milk clotting test in the same way as that described by Herriott for pepsin (8), trypsin activity by a new spectrophotometric method, worked out by Kunitz (9), and pepsin activity as described by Anson (7), with the modification that tyrosine and tryptophane were measured spectrophotometrically.

To make a comparison with Sizer's results more convenient our results have been recalculated to a common basis of 25 mg. of protease preparation. This recalculation involves only the multiplication by the ratio of the amount of proteolytic enzyme preparation used by Sizer and that used by us.

The results are presented in Table I and Fig. 1 but require some comments.

In the case of chymotrypsin there was a slow uptake of oxygen which

Incubation of 25 Mg. of Chymotrypsin, Trypsin, and Pepsin Preparations at pH 7.3 and 37°, with and without Tyrosinase

Protease preparation	Reaction time*	Total oxygen uptake	Non protein tyrosine and tryptophane in absence of tyrosinase, in mg tyrosine		Per cent loss in proteolytic activity
			Initial	Final	
	hrs.	*microliters*			
Chymotrypsin	4½	72	0.28	0.47	0
Trypsin I	9	320	0.42	3.05	100
Pepsin I	6½	202	0 95	1 00	100
" II	8	60	0.11	0.23	

* In the reaction time is included the equilibration time prior to the addition of the tyrosinase
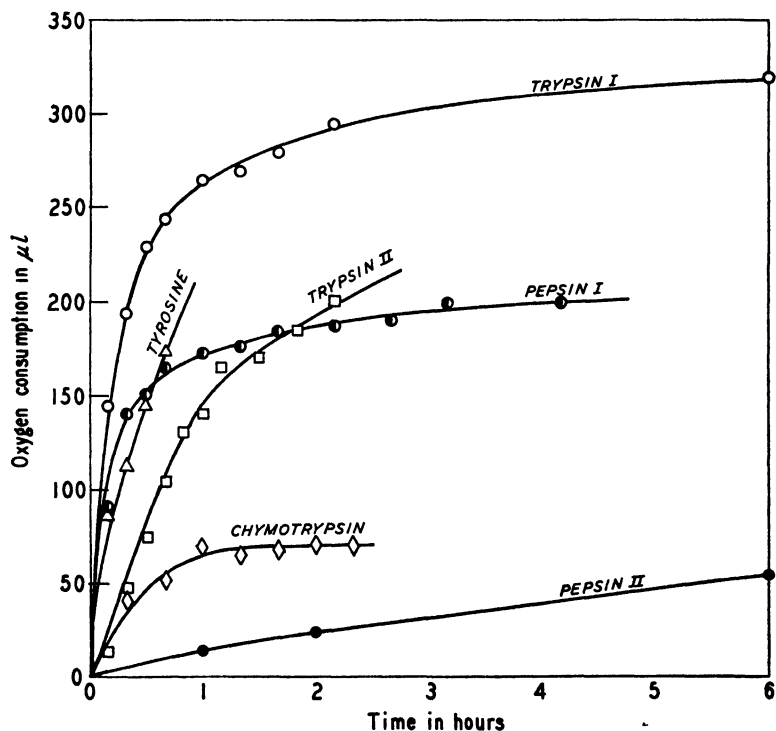
FIG. 1. Oxygen uptake catalyzed by tyrosinase of preparations of crystalline chymotrypsin, trypsin, pepsin, and tyrosine. Each curve represents the oxygen uptake of 25 mg. of enzyme preparation in the presence of about 1 ml. of tyrosinase solution. The tyrosine curve represents 2.5 mg. of tyrosine oxidized by 0.1 ml. of tyrosinase solution.

ceased after about 1 hour. In the starting material there was present a small amount of non-protein tyrosine and tryptophane, which showed a slight increase during the experiment. No decrease could be observed in the proteolytic activity as compared with the starting material.

The results with trypsin (Trypsin I, Table I and Fig. 1) were quite different. A considerable oxygen uptake was observed. There was also a formation of large amounts of non-protein tyrosine and tryptophane during the experiment. Further, no proteolytic activity was left after the 9 hours duration of the experiment. A certain amount of this non-protein tyrosine and tryptophane must have been formed during the equilibration time of 3 hours prior to the addition of the tyrosinase. In order to escape this preliminary decomposition of trypsin the experiment was modified in that, during the equilibration time, the acidified trypsin solution was kept in the side arm instead of the tyrosinase solution. As may be seen (Trypsin II, Fig. 1) the initial rate of oxygen uptake was markedly slower in this case.

The pepsin preparation also took up large amounts of oxygen when treated with tyrosinase (Pepsin I, Table I and Fig. 1). The starting material contained considerable amounts of non-protein tyrosine and tryptophane. No proteolytic activity was left at the end of the experiment. It was found, however, that this loss in activity occurred almost instantaneously after the neutralization of the acidified stock solution of pepsin. This was to be expected, since swine pepsin is known to be rapidly destroyed under the conditions of the experiment (10).

Denatured pepsin was prepared from native pepsin by adjusting the pH of its solution to 7.3. The denatured pepsin was then freed from the main part of the contaminating non-protein tyrosine by reprecipitating the protein several times at pH 4, at which the denaturated protein has a minimum of solubility. This preparation was then subjected to the treatment with tyrosinase. As may be seen (Pepsin II, Table I and Fig. 1), the preparation had then lost by far most of its ability to be oxidized by tyrosinase. In spite of the purification the denaturated pepsin still contained a small amount of non-protein tyrosine and tryptophane and an additional small amount was formed during the incubation.

<center>DISCUSSION</center>

From the results obtained certain facts appear, many of which have been known for a long time. In the first place it is shown that all the enzyme preparations tested contain a greater or lesser amount of protein split-products. This is generally the case when no special precautions are taken to avoid it. It is also demonstrated in the case of pepsin that when the main part of the non-protein tyrosine is removed the capacity of the

enzyme preparation to be oxidized by tyrosinase is also mainly lost. Consequently the inference must be drawn that the main substrate for the tyrosinase is the protein split-products present. Actually we have not been able to obtain unequivocal evidence of the action of tyrosinase on any of the proteases investigated.

In the case of trypsin the situation is more complicated in that this enzyme is unstable at pH 7.3 (11) and is rapidly autolyzed during the incubation, with the formation of non-protein tyrosine. When, however, trypsin is incubated for several hours at pH 7.3 prior to the addition of tyrosinase, the initial rate of oxidation by tyrosinase is much faster, which also strongly indicates that it is the split-products containing tyrosine which are oxidized.

The behavior of chymotrypsin is not in conflict with the explanation proposed. The small oxygen uptake might well be explained by amounts of non-protein tyrosine present.

It has been claimed by Sizer that the proteolytic activities of trypsin and pepsin were not decreased during the incubation with tyrosinase at pH 7.3. This statement seems difficult to reconcile with the well established fact that crystalline trypsin is very unstable at this pH in the absence of its substrate (11). Further, it has been known for a long time that pepsin is irreversibly, completely, and almost instantaneously inactivated at a neutral reaction (10, 12, 13). These circumstances were confirmed in our experiments in which we found a complete loss in proteolytic activity of these enzymes after the incubation. The main cause of these divergent results must be that Sizer in his activity measurements used only the enzyme solutions incubated with inactive tyrosinase as controls, whereas we in addition used the original, untreated enzyme solution for the same purpose. The enzyme solutions incubated with boiled tyrosinase, however, are insufficient controls, as they have undergone a profound decomposition which is quite independent of the presence or absence of tyrosinase.

SUMMARY

The action of tyrosinase on chymotrypsin, trypsin, and pepsin has been studied. The start of the reaction depends on the purity of the protease preparation used. Preparations which contain non-protein tyrosine absorb considerable quantities of oxygen, whereas in those which have been freed from most of the non-protein tyrosine there is much less reaction. Some decomposition occurs during the course of the reaction itself, especially in the case of trypsin; so that it is difficult to say with certainty that no reaction occurs with the protein, although such a reaction seems unlikely.

Pepsin is instantly inactivated at the pH of the reaction mixture; so

that no conclusion as to the effect of tyrosinase on the activity of this enzyme is possible.

The author wants to thank Dr. J. H. Northrop for suggesting this work and for many helpful discussions.

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A NEW SYNTHESIS OF DJENKOLIC ACID

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Several years ago considerable interest was aroused by the discovery by van Veen and Hyman (1) of a new naturally occurring sulfur-containing amino acid which they had isolated from the djenkol bean (*Pithecolobium lobatum*) and named djenkolic acid. They showed that it was probably the cysteine thioacetal of formaldehyde. This structure was confirmed by du Vigneaud and Patterson (2) who synthesized the *l*-cysteine thioacetal of formaldehyde by the condensation of methylene chloride with 2 moles of *l*-cysteine in liquid ammonia and showed their synthetic compound to be identical with the naturally occurring djenkolic acid.

Van Veen and Hyman suggested that the natural synthesis of djenkolic acid in the plant might occur by a condensation of formaldehyde and cysteine. Such thioacetals were apparently not formed by condensation of these compounds under conditions studied at that time or later by various workers (3–6). Whenever aldehydes or ketones were caused to react with cysteine, the product was either an addition compound (I) or a thiazolidinecarboxylic acid (II).

$$
\begin{array}{cc}
\underset{\underset{\displaystyle R'}{\overset{\displaystyle |}{|}}}{\overset{\overset{\displaystyle OH}{|}}{R-C}}-S-CH_2-\underset{}{\overset{\overset{\displaystyle NH_2}{|}}{CH}}-COOH
&
\begin{array}{c}
R \quad\; S\!-\!\!-\!\!-CH_2 \\
\diagdown \diagup \qquad\;\; | \\
C \qquad\qquad | \\
\diagup \diagdown \qquad\;\; | \\
R' \quad NH\!-\!CH\!-\!COOH
\end{array}
\\
(I) & (II)
\end{array}
$$
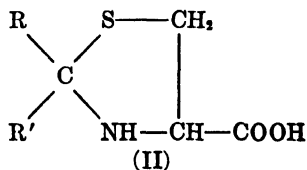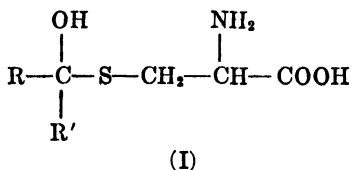
A further study of the reaction of aldehydes and ketones with cysteine has led us to the synthesis of djenkolic acid by the direct combination of 2 moles of cysteine and 1 mole of formaldehyde in a strongly acid solution. This condensation is best effected by adding 1 mole of formaldehyde (40 per cent formalin) to a concentrated solution of 2 moles of cysteine in 7 N hydrochloric acid at room temperature. The reaction proceeds with moderate speed at room temperature but may be hastened by warming the reaction mixture to 70°. The identity of the product with djenkolic acid was shown by a comparison of its physical and chemical properties and those of three of its derivatives with samples made from synthetic djenkolic acid prepared by the method of du Vigneaud and Patterson (2).

Schubert (3) studied the reaction of formaldehyde and cysteine in a

buffered solution and found that 4-thiazolidinecarboxylic acid was formed, and Ratner and Clarke (4) measured the rate of its formation in solutions with a pH between 1.5 and 12, finding that the rate was measurable up to pH 5; at a higher pH than this, the reaction proceeded too fast to be measurable. In the present study it was found that the formation of djenkolic acid proceeded to a negligible amount in acid less concentrated than 5 N.

Ratner and Clarke showed that in the formation of the thiazolidine-carboxylic acid the reaction proceeded by an initial reaction of formaldehyde with the sulfhydryl group of cysteine, followed by elimination of water with ring closure to give 4-thiazolidinecarboxylic acid. In the present study, it has been found that the addition of 1 mole of formaldehyde to 1 mole of cysteine in 6 N HCl likewise results mainly in the formation of 4-thiazolidinecarboxylic acid. In the presence of an excess of cysteine, djenkolic acid is mainly formed. Preliminary experiments have indicated that the thiazolidine formation in the absence of an excess of cysteine takes place at a much slower rate than the formation of djenkolic acid in the presence of an excess of cysteine. The formation of djenkolic acid may proceed stepwise with an initial rapid addition of cysteine to formaldehyde to give a hemithioacetal, followed by a slower addition of a 2nd molecule to form djenkolic acid.

$$
\begin{array}{ccc}
& \overset{\displaystyle NH_2 \cdot HCl}{|} & \overset{\displaystyle NH_2 \cdot HCl}{|} \\
HCHO\ +\ HSCH_2CHCOOH & \longrightarrow & HOCH_2SCH_2CHCOOH
\end{array}
$$

$$
\overset{\displaystyle NH_2 \cdot HCl}{HSCH_2\overset{|}{C}HCOOH} \qquad -H_2O
$$

$$
\begin{array}{cc}
\overset{\displaystyle NH_2 \cdot HCl}{SCH_2\overset{|}{C}HCOOH} & S\!\!-\!\!CH_2 \\
CH_2 & CH_2 \qquad | \\
SCH_2CHCOOH & NH\!-\!CH\!-\!COOH \\
\overset{|}{NH_2 \cdot HCl} &
\end{array}
$$

EXPERIMENTAL

Preparation of Djenkolic Acid—To a solution of 10.0 gm. of *l*-cysteine hydrochloride in 20 cc. of 6 N HCl were added 2.6 cc. of 40 per cent formalin. The resulting solution was warmed to 70° and allowed to cool to room temperature and stand overnight. It was then neutralized by the addition

of 6 N NaOH solution and the precipitated djenkolic acid was collected and washed with water.   This material, after standing in a sodium cyanide solution, gave a faint positive test for the sulfhydryl group when treated with sodium nitroprusside.   To remove the small amount of cystine contamination, the moist acid was suspended in 100 cc. of water and concentrated ammonium hydroxide was added dropwise until all of the material dissolved; 1.5 gm. of sodium cyanide were added and the resulting solution was allowed to stand at room temperature for 30 minutes.   It was then made neutral to litmus by the addition of glacial acetic acid (*hood*) and the precipitated acid was collected and washed twice with water.   It was recrystallized by suspending it in 100 cc. of water, heating to boiling, and adding 6 N HCl dropwise until the material just dissolved. The hot solution was filtered and then carefully neutralized by the addition of 6 N NaOH with vigorous stirring at the boiling point.   The suspension was cooled and filtered, and the product was washed three times with water and dried *in vacuo* at 95°.   The yield was 5.5 gm. (68 per cent of the theoretical amount) of a product which gave a negative test for the sulfhydryl and disulfide groups and for the chloride ion.   The compound did not exhibit a definite melting point but gradually decomposed from 300–350°; $[\alpha]_D^{20.5} = -65.0°$ for a 1 per cent solution in 1 N HCl; $[\alpha]_D^{25} = -47.5°$ for a 2 per cent solution in 1 per cent HCl.

The solubilities, crystal form, decomposition point, and rotations of this product agree within experimental error with the corresponding properties of a sample of known djenkolic acid prepared by condensing methylene chloride and *l*-cysteine in liquid ammonia.

$C_7H_{14}O_4N_2S_2$ Calculated.   C 33 06, H 5 55, N 11 02, S 25.21
254.3   Found   " 33 38, " 5 58, " 11 10, " 25 43

Monohydrochloride of Djenkolic Acid—The monohydrochloride was prepared by dissolving djenkolic acid in hot 1 per cent HCl and cooling the solution; it crystallized in slender prisms tending to form radiating clusters.   After three recrystallizations from 1 per cent HCl, the product was dried *in vacuo* over phosphoric anhydride; m p. 250–300° (with decomposition).[1]

$C_7H_{15}O_4N_2S_2Cl$ Calculated.   Cl 12 19, S 22.05
290 8   Found   " 12 35, " 22.50

Dibenzoyldjenkolic Acid—This derivative was prepared according to the directions of du Vigneaud and Patterson (2), recrystallized four times from 50 per cent methanol, and dried at 70° *in vacuo* over phosphoric anhydride.   The sample melted to a milky liquid at 87.5–89° (capillary);

[1] All melting points are corrected micro melting points, unless otherwise specified.

a sample of the dibenzoyl derivative prepared from authentic djenkolic acid exhibited the same behavior and admixture of the two samples caused no depression in the melting point.

$C_{21}H_{22}O_6N_2S_2$. Calculated. N 6.06, S 13.86
462.5 Found. " 6.12, " 13.94

TABLE I

Effect of Concentration of Acid upon Yield of Djenkolic Acid

HCl	Djenkolic acid	Yield
N	*mg.*	*per cent*
0.5	0	0
1	0	0
2	17	4
3	66	17
4	71	18
5	166	42
6	250	63
7	273	69
8	260	66
11	260	66

TABLE II

Rate of Formation of Djenkolic Acid

Time	Djenkolic acid	Yield
hrs.	*mg.*	*per cent*
0.17	Trace	+
0.35	3	1
0.50	16	6
1.5	50	18
2.0	64	23
2.5	88	32
3	99	36
6	119	43
24	186	67
48	182	66

Dihydantoin of Djenkolic Acid—This derivative was prepared according to the directions of van Veen and Hyman (1). It melted at 201–204°, and no depression in melting point was observed when it was mixed with a sample prepared from authentic djenkolic acid.

Effect of Concentration of Acid upon Yield of Djenkolic Acid—To solutions of 0.5 gm. of *l*-cysteine hydrochloride in 1.0 cc. of HCl of the normality specified in Table I was added 0.14 cc. of 40 per cent formalin. The

solutions were heated to 70° and allowed to cool to room temperature and stand overnight. They were then neutralized by the addition of 6 N NaOH. All samples were made up ·to the same volume and the djenkolic acid was collected, washed, and dried *in vacuo*.

Rate of Condensation of Formaldehyde and Cysteine in 6 N Acid. 2 Moles of Cysteine and 1 Mole of Formaldehyde—A solution of 5.0 gm. of *l*-cysteine hydrochloride in 10.0 cc. of 6 N HCl was prepared, 1.4 cc. of formalin were added, and the volume was made up to 15.0 cc. with 6 N acid. 1 cc. aliquots were removed at intervals, neutralized with 6 N NaOH, and the precipitated djenkolic acid was collected, washed, and dried. The results are shown in Table II.

1 Mole of Cysteine and 1 Mole of Formaldehyde—To a solution of 1.0 gm. of *l*-cysteine hydrochloride in 2 cc. of 6 N HCl was added 0.56 cc. of formalin. The solution became hot spontaneously and was then allowed to cool to room temperature and stand overnight, whereupon a considerable amount of crystalline 4-thiazolidinecarboxylic acid hydrochloride separated. The mixture was cooled in an ice bath and the product was collected on a sintered glass filter, washed with cold 6 N HCl, and dried *in vacuo*. A yield of 0.75 gm. (70 per cent of the theoretical amount) was obtained; $[\alpha]_D^{20.5} = -78.5°$ for a 1 per cent solution in 1 N HCl. An additional amount could be obtained by concentrating the mother liquors. A small amount of the hydrochloride was dissolved in water, the solution was made neutral by the addition of 1 N NaOH, and the precipitated 4-thiazolidinecarboxylic acid was collected and recrystallized from a small amount of water. The melting point was 201–205° (with decomposition); no depression was observed in the melting point of a mixture of this compound with an authentic sample of 4-thiazolidinecarboxylic acid prepared according to the method of Schubert (3).

SUMMARY

Djenkolic acid has been prepared by the direct combination of 1 mole of formaldehyde with 2 moles of *l*-cysteine in a strongly acid solution.

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THE DEPOSITION AND ANTIOXYGENIC BEHAVIOR OF
α-, β-, AND γ-TOCOPHEROLS IN RAT FATS*

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It has been shown previously that tocopherols obtained from the diet are the only true inhibitors of autoxidation that occur normally in the rendered abdominal fats of rats (1). By eliminating tocopherols from the diet, it is possible to produce rats whose fats, when rendered, are essentially free of primary antioxidants and show a very low stability. A quantitative technique based on these observations and on oxygen absorption measurements on the rendered fats at 100° was devised and used in studying the deposition and storage of α-tocopherol in adipose tissues (2). It was shown that, as storage sites of α-tocopherol, the fat depots have an even greater capacity than the liver, whose importance in this rôle has been reported by other investigators (3, 4).

There have been apparent differences between the observations of various investigators on the relationships between the tocopherol contents of various tissues and organs, probably as a result of differences in various experimental factors. Because of these differences it appears to be particularly desirable (*a*) to obtain data concerning the deposition of tocopherols in various tissues with the tocopherols individually and (*b*) to obtain such data at various known intakes of tocopherol.

In the present study, therefore, the same experimental technique described earlier (2) was used to obtain data concerning the relative amounts of synthetic α-, β-, and γ-tocopherol stored in the abdominal fat tissues exclusive of the mesentery in the rat under various conditions. Data were also obtained, with an analytical technique based on micro measurements of peroxides rather than oxygen absorption measurements, which qualitatively compare the depositions of synthetic and natural α- and γ-tocopherols in abdominal, skin, and ham fats.

Deposition of Synthetic α-, β-, and γ-Tocopherols in Abdominal Fats[1]

Experimental—Details of the methods used in producing vitamin E-deficient rats, the diets, the rendering of the abdominal fats, and the measurements of oxygen absorption were described in previous papers (1, 2).

Repeated experiments showed that the iodine numbers of the fats from different rats in each complete study varied only slightly. The effects of the minor differences in composition on the relative autoxidation characteristics of different samples of the same tissue fat, therefore, have been ignored in all that follows.

Chemical assays by a modification of the Furter-Meyer method (5) showed the α-tocopherol to be 90 per cent pure and the γ-tocopherol to be 53 per cent pure. No direct analysis of the β-tocopherol was attempted, but its purity was reported to be approximately 90 per cent. In the absence of a chemical analysis such as was made for the α- and γ-tocopherols, few quantitative interpretations of the data for β-tocopherol will be made. The data are included because quantitative interpretations may be made at some future time when accurate antioxygenic indexes for the pure tocopherols in rat fat become available. With the aid of such indexes, an indication of the purity of the β-tocopherol may be obtained from the data in Fig. 1.

Relative Antioxygenic Activities of Synthetic α-, β-, and γ-Tocopherols—Solutions of the three tocopherols at various concentrations in vitamin E-free rat fat were prepared. Arbitrarily defined induction periods were measured in a Warburg oxygen absorption apparatus at 100°, with 0.4 ml. samples of fat in 70 ml. flasks. The data have been plotted in Fig. 1. Each point represents the average of at least three oxygen absorption measurements. In the case of the samples containing α- and β-tocopherols, there was a period of very low oxygen uptake, followed by a sudden and sharp increase in the rate of absorption. The major part of the sudden increase occurred within an interval of 10 minutes, which was the time interval between manometer readings. The induction period, therefore, could be conveniently defined by the mid-point in the interval during which the sudden increase took place. In the case of γ-tocopherol, however, the increase in absorption was not so abrupt and it was necessary to use a still more arbitrary method in defining the induction period. For this purpose, it was convenient to select that point at which the rate of fall in oxygen pressure in the flasks became the equivalent of 1 mm. height of manometer fluid (butyl phthalate) per minute.

Probably the less abrupt termination of the induction period in the case of γ-tocopherol is attributable to some characteristic of the mechanism of

[1] The synthetic β- and γ-tocopherols were generously supplied by Merck and Company, Inc., through the courtesy of Dr. D. F. Robertson. The synthetic α-tocopherol used was also a Merck product.

antioxidation peculiar to γ-tocopherol. Golumbic (6) has attributed the gradual increase in the rate of oxidation of vegetable oils to the successive actions of two antioxidants, tocopherol and chroman-5,6-quinone. Swift *et al.* (7) have shown that chroman-5,6-quinone is produced from γ-tocopherol in the autoxidation of fats and that, although the antioxygenic activity of the quinone is generally slight, nevertheless it does exert a definite antioxygenic effect in animal fats.

A second explanation of the more gradual increase in the oxygen uptake with γ-tocopherol could be based on the presence of antioxygenic impurities in the synthetic γ-tocopherol. This explanation is made improbable by
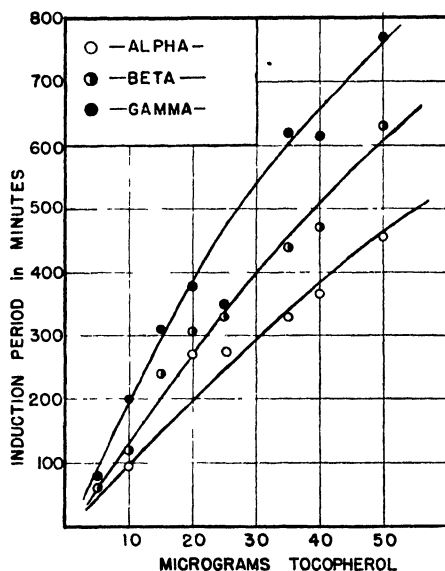
Fig. 1. Induction periods of vitamin E-free abdominal fats to which various concentrations of tocopherols have been added

the observation that the rendered fat from vitamin E-deficient rats which had been fed γ-tocopherol behaves in identically the same manner. Hanson *et al.* (8) have shown that of a group of seven antioxidants, which included α- and γ-tocopherols, only the tocopherols were deposited in the abdominal fats. It is not likely that the γ-tocopherol and the impurities associated with it would be deposited in unchanged proportions in the fat tissues.

From Fig. 1, it is seen that at low concentration the induction periods are almost straight line functions of the concentration. The ratios of the antioxygenic potencies of these samples of α-, β-, and γ-tocopherols were about 3:4:5.

Relative Amounts of Tocopherols Deposited in Abdominal Fat Tissues—
Individual solutions of the three tocopherols in the ethyl esters of lard acids
were made, containing 25 and 50 mg. of tocopherol per ml. The six solu-
tions so prepared were fed by stomach tube in single 1 ml. doses to vitamin
E-deficient rats. Groups of two to four rats were sacrificed at various in-
tervals thereafter. Column 5 in Table I lists the induction periods of the
rendered abdominal fats obtained under the various experimental conditions
given in Columns 1 to 4. Column 6 indicates the equivalent tocopherol
concentrations as obtained from the reference curves in Fig. 1, based on

TABLE I

Relative Amounts of Tocopherols Deposited in Abdominal Fat Tissues

Tocopherol	Dose fed	Sex	Time between feeding and sacrifice	Induction period of rendered fat	Tocopherol per gm fat
	mg		*days*	*min*	*γ*
α-	50	F.	8	380	39
	50	M.	8	330	33
	50	Mixed	15	300	30
	25	F.	8	214	21
	25	"	15	248	24
β-	50	M.	2	265	20
	50	"	10	303	23
	50	"	15	420	32
	25	F.	8	183	14
	25	"	15	266	20
γ-	50	M.	2	80	3.5
	50	Mixed	8	138	6 5
	50	M.	10	80 (?)	3 5
	50	"	15	150	7 0
	25	F.	8	115	5 0
	25	"	15	91	4 0
Controls	0	"		10	<2 (α-)
		M.		10	<2 "

the assumption that the tocopherols were 100 per cent pure. Actually,
since the curves in Fig. 1 are approximately straight lines in the regions con-
cerned, and since at the lower concentrations the amounts of tocopherol de-
posited are (very roughly) proportional to the amounts fed, the figures given
in Column 6 are roughly the same as those that would have been obtained if
tocopherols of 100 per cent purity had been fed in the amounts indicated in
Column 2. This statement is, of course, based on the assumption that the
impurities in the samples of tocopherols were relatively inert biologically
and as antioxidants, in so far as these results are concerned.

The conversion of the induction periods in Column 5 to the equivalent

tocopherol concentrations in Column 6 was shown to be justified in an earlier paper in this series on the basis that the tocopherol concentrations so obtained agreed very well with the values that were obtained by direct chemical analysis with the analytical method devised by Chipault *et al.* (5).

It was previously reported that a maximum concentration of α-tocopherol in the abdominal fat depots occurred approximately 7 to 10 days after feeding a single 50 mg. dose (2). Table I suggests that the synthetic β-tocopherol, whether fed in 25 or 50 mg. doses, required a somewhat longer time to reach a maximum deposition in the abdominal fats. The data for γ-tocopherol on this point are inconclusive. Of the two doses fed in each case, the larger dose led to the deposition of a greater concentration in the fats.

When the tocopherols are fed in equal doses, the results indicate that γ-tocopherol, in spite of its greater antioxidant activity, produces a less stable fat than either α- or β-tocopherols. Similar observations on the relative behaviors of α- and γ-tocopherols were reported in a previous study (8). With these stabilities converted into tocopherol concentrations, it is found that α- and β-tocopherols are deposited in significantly greater amounts than γ-tocopherol.

Deposition of Synthetic and Natural α- and γ-Tocopherols in Abdominal, Skin, and Ham Fats[2]

Experimental—On the basis of the previous results, it was felt desirable to compare the behavior of synthetic and natural tocopherols and to extend the studies to fats other than the abdominal fats. In these experiments, four groups of three vitamin E-deficient rats were fed 25 mg. doses of synthetic and natural α- and γ-tocopherols by stomach tube. The dosages were dissolved in 1 ml. of fresh lard, and control animals were given 1 ml. of fresh lard containing no added tocopherols. After 10 days the rats were sacrificed and the abdominal fats removed and rendered. 0.1 gm. portions of each sample were weighed into a series of small glass vessels, 10 mm. in diameter and 8 mm. deep. The vessels were then stored in an oven at 63°. From time to time a vessel was removed and an analysis of the peroxide value of the contents made.

The fat in skin and muscle tissue was also studied. The skin and hams were stored at 63° and at intervals a portion of tissue was removed and ground with sand in a mortar. The fat was then extracted with chloroform. After the chloroform solution had been filtered, evaporated to dry-

ness on a water bath, and weighed, the peroxide value was measured in the same manner as in the abdominal fat.[*]

A micromethod was applied in determining the peroxide values. The 0.1 gm. samples of fat were dissolved in 2.5 ml. of 3:2 mixture of acetic acid and chloroform in a 50 ml. Erlenmeyer flask; 0.05 ml. of a saturated aqueous solution of KI was added, followed after 1 minute by 5 ml. of distilled water. The samples were titrated with 0.01 N sodium thiosulfate, with a 5 ml. micro burette graduated in divisions of 0.01 ml. The results by this method checked very closely with those obtained by the macro-method of Wheeler (9), as modified by Lea (10).

Antioxygenic Activities of Synthetic and Natural α- and γ-Tocopherols upon Direct Addition and after Feeding—Fig. 2 gives the peroxide accumula-
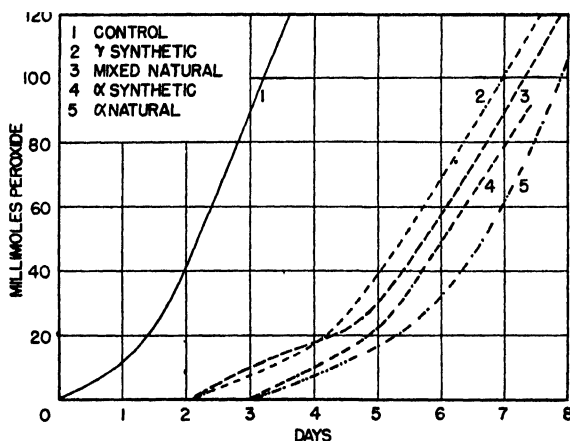
FIG. 2. Peroxide accumulation in samples obtained by adding tocopherols to vitamin E-free fat (2 γ per gm.).

tion curves for the vitamin E-free abdominal fats to which direct addition of 2 γ per gm. of the various samples of tocopherol had been made. It is notable in Fig. 2 that synthetic γ-tocopherol stabilized the fat to a lesser extent than either the synthetic or natural α-tocopherol, unlike the earlier results of the oxygen absorption measurements. The differences at this low concentration are approximately the same in magnitude as the limits of experimental error of the data in Fig. 1 at tocopherol concentrations of 2 γ per gm. However, it is believed that the differences are not due entirely to experimental error, but are partly attributable to the considerable differences in the two methods of measurement. Other instances

[*] We are indebted to Dr. H. C. Black for suggestions on the preparation of tissue fat samples.

have been observed in this laboratory and elsewhere, in comparing two antioxidants of similar antioxygenic potencies, where the more potent antioxidant by one method of measurement was the less potent by the other method. Another possible factor that should be pointed out is that the
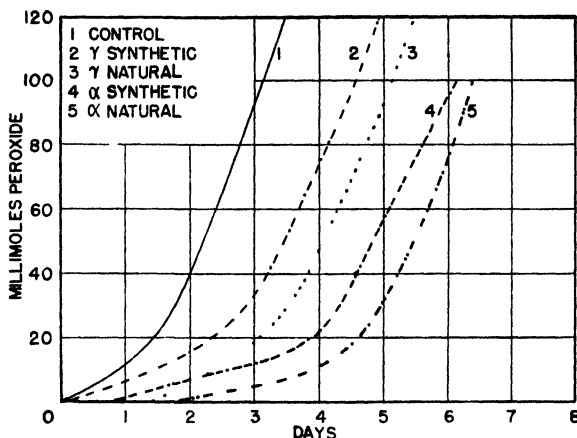
FIG. 3. Peroxide accumulation in rendered abdominal fats from rats sacrificed 10 days after feeding a 25 mg. dose of tocopherol.
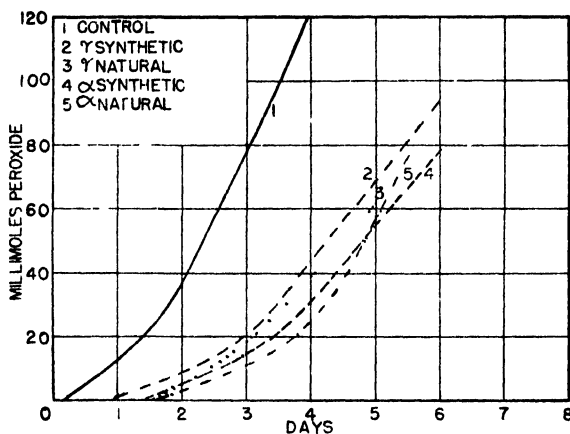
FIG. 4. Peroxide accumulation in fat in skin

synthetic tocopherols used in this case were not the same samples as were used in the earlier studies.

In Figs. 3 to 5, the peroxide values of the various samples of fats from animals that had been fed tocopherols are plotted against time. In none of these cases was there any sudden and sharp increase in the rate of peroxide

accumulation during the course of the oxidation as there was in the rate of oxygen absorption in the earlier samples that contained α- and β-tocopherols. Nevertheless, the data concerning the accumulation of peroxides yield smooth curves, which are sufficiently accurate to make semiquantitative comparisons possible. The induction periods, for purpose of comparison, are arbitrarily defined as the length of time required for the accumulation of 20 mM of peroxide per kilo. These induction periods may not be compared with those previously obtained by oxygen absorption measurements, since the conditions used in the two types of measurements are greatly different.

Since Fig. 2 contains data for only one concentration of added tocopherol, it is not possible to make any quantitative estimates of the amounts of
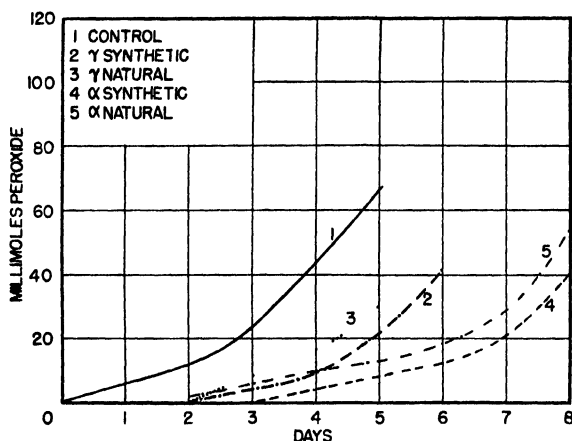
FIG. 5. Peroxide accumulation in fat in hams

tocopherol represented in the curves in Figs. 3 to 5. Certain qualitative comparisons may be made, however.

In the samples of vitamin E-free fats to which the tocopherols were added directly, and in the samples of abdominal depot fats, the natural tocopherols gave slightly longer induction periods than the corresponding synthetic tocopherols. This could be due to such possible causes as differences in the antioxygenic indexes of the natural and synthetic tocopherols, differences in their efficiency of absorption and deposition, and differences in purity. In any case the differences are not large, and the results indicate that natural and synthetic tocopherols are much alike in their antioxygenic activities and in the amounts deposited in the abdominal fats. In the case of skin and ham fats the differences between synthetic and natural tocopherols are still less marked.

In all cases in Figs. 3 and 5, induction periods of the fats from animals that had been fed α-tocopherol were greater than the induction periods of those animals that had been fed γ-tocopherol.   γ-Tocopherol is apparently deposited to a considerably lesser extent than α-tocopherol in ham fats as well as in the abdominal fats.   In skin fats, the different forms of tocopherol were much alike in the magnitude of their stabilizing effects.

The natural tocopherols used in this study were more than 90 per cent pure; the synthetic tocopherols were somewhat less pure, but, as before, the conclusions drawn regarding the relative amounts of tocopherol deposited are valid if one may assume that the impurities in the synthetic tocopherols do not exert any appreciable antioxygenic or prooxygenic effects.

DISCUSSION

It is of interest to compare the fat stability data in Column 5 of Table I with the relative biological potencies of the tocopherols, as reported by various investigators.   Karrer et al. (11), Mason (12), and Joffe and Harris (13) all report that the biopotency of β-tocopherol is one-half or less of that of α-tocopherol.   Assuming that the synthetic α- and β-tocopherols were of the same degree of purity, the stabilizing effects of equal doses of the two tocopherols on the abdominal fats were roughly equal 8 and 15 days after feeding.   It should be pointed out that these amounts of tocopherol are far in excess of the median fertility doses.   The comparison is therefore based upon the assumption that the same relative stabilizing effects will be obtained on feeding much lower doses.   This assumption is supported by a comparison of the effects of 25 and 50 mg. doses in Table I.

The relative biopotency of γ- compared with α-tocopherol is given variously as one-third to one-fifth by Karrer et al. (11), as one-fourth by Mason (12), and as one-twelfth by Joffe and Harris (13).   The 25 mg. dose of γ-tocopherol is not far in excess of the median fertility dose.   There appears to be no direct connection between the relative induction periods and any of the above figures for the relative biopotencies.

The amounts of the various tocopherols deposited in the fat depots, recorded in Column 6, are roughly proportional to the biopotencies, particularly to those given by Karrer et al., although the relative biopotencies obtained by Joffe and Harris, who used highly purified natural tocopherols, are probably the more accurate.   The data for the natural tocopherols, in Figs. 2 to 5, are not sufficiently quantitative to make possible any estimates of the actual amounts of tocopherol deposited, but nevertheless permit the conclusion that the deposition of the natural tocopherols approximately parallels the deposition of the synthetic tocopherols.   It ap-

pears likely that there is no simple direct relationship between the biopotencies of the tocopherols and their antioxygenic properties in rendered animal fats. The data indicate that there may be a relationship between the biopotencies and the amounts deposited in fatty tissues.

SUMMARY

The amounts of synthetic α-, β-, and γ-tocopherols deposited in abdominal fats have been compared by means of measurements of the autoxidative behavior of the rendered fats. Even when allowing for possible differences in the degree of purity of the samples, it was found that synthetic γ-tocopherol was deposited in much smaller amounts than either the synthetic α- or β-tocopherol, the amounts of the latter two being more nearly alike. As with the α-tocopherol, the β- and γ-tocopherols appeared not to reach their maximum depositions until 7 or more days after feeding, and possibly not until 15 days in the case of the β-tocopherol.

In the oxygen absorption measurements at 100°, samples of abdominal fat containing γ-tocopherol showed a more gradual increase in the rate of oxygen uptake at the end of the induction period than did samples containing α- and β-tocopherols. This behavior appears to be an inherent property of γ-tocopherol, possibly being attributable to the development of an oxidation product which is in itself somewhat antioxygenic.

Further comparisons were made of the deposition of synthetic and natural α- and γ-tocopherols in abdominal, skin, and ham fats, based on studies of the peroxide accumulation in these fats at 63°, with a micromethod for the determination of peroxides. It was found that α-tocopherol was deposited to a greater extent than γ-tocopherol in all cases, except in the case of skin fat. The evidence, although not conclusive, indicates that the natural forms of these tocopherols were not greatly different from the synthetic, either in their antioxygenic characteristics or in the amounts deposited in fatty tissues.

The results indicate that the biopotencies of tocopherols are not directly related to their effects upon the autoxidation characteristics of the rendered fats in which they are deposited by feeding. The results suggest that a relationship may exist between the biopotencies and the relative amounts of tocopherols deposited in various body fats or other tissues.

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LETTERS TO THE EDITORS

STREPTOMYCIN B, AN ANTIBIOTICALLY ACTIVE CONSTITUENT OF STREPTOMYCIN CONCENTRATES

Sirs:

We have isolated from streptomycin concentrates the crystalline reineckate of an antibiotically active base, which in view of its close similarity to streptomycin, has been designated as streptomycin B.

The application of the chromatographic method in the purification of streptomycin according to Carter et al.[1] yields in addition to active fractions of high streptomycin content a number of less active, more firmly adsorbed fractions, ranging in potency from 150 to 200 units per mg. The presence in such fractions of a substance other than streptomycin, capable of forming maltol in alkaline medium, was inferred from the following observations: (1) These fractions on treatment with alkali yielded 2 to 3 times the quantity of maltol expected from their biological potency. (2) The application to such fractions of the Craig counter-current distribution technique, as adapted to streptomycin in this laboratory,[2] afforded distribution curves which demonstrated clearly that "streptomycin B" was the major maltol-forming component present.

The isolation of streptomycin B in pure form was accomplished by fractional crystallization of the reineckates obtained from the above chromatographic fractions. This procedure effected the removal of streptomycin and other contaminants by two or three crystallizations, while fractional crystallization of the helianthate did not lead to a streptomycin-free product. The reineckate of streptomycin B crytallizes in large, thin plates, which contain water of crystallization (8.10 per cent). Its distribution curve[2] is in good agreement with the theoretical curve for $K = 0.44$. The anhydrous substance melts at 178–179° (decomposition, corrected) and shows the following analytical figures: C 26.89, 27.21, H 4.24, 4.53, N 20.1, S 22.22, Cr 8.70, 9.25. Its activity in broth test against *K. pneumoniae*[3] is about 100 streptomycin units per mg. The hydrochloride of strepto-

[1] Carter, H. E., Clark, R. K., Jr., Dickman, S. R., Loo, Y. H., Skell, P. S., and Strong, W. A., *J. Biol Chem.*, **160**, 337 (1945).

[2] Titus, E., and Fried, J., *J. Biol. Chem*, **168**, 393 (1947).

[3] Donovick, R., Hamre, D., Kavanagh, F., and Rake, G, *J. Bact.*, **60**, 623 (1945).

mycin B was obtained as a white, amorphous powder by decomposition of the reineckate with silver sulfate and back titration with barium chloride. The vacuum-dried substance decomposes at 179–182° (corrected) and shows the following analysis: C 35.75, H 6.22, N 10.9, Cl 12.4. It may be noted that while the absolute values for nitrogen and chlorine are approximately 20 per cent lower than the corresponding values for streptomycin trihydrochloride the atomic ratio N:Cl is close to 7:3 as in streptomycin. $[\alpha]_D^{25} = -47°$, (c, 1.35 in water). The titration curve of the hydrochloride shows the presence of a weakly basic group (pKa', 7.6; equivalent weight, 780). Its antibiotic activity against $K.$ $pneumoniae$ is 190 units per mg.; that is, about one-fourth of the activity of streptomycin trihydrochloride. In preliminary measurements[4] it was found that from 4 to 8 times more streptomycin B than streptomycin was required to inhibit four other test organisms.

Streptomycin B is inactivated by carbonyl reagents at room temperature. On catalytic hydrogenation it absorbs approximately 1 mole of hydrogen and forms dihydrostreptomycin B, which is inert to carbonyl reagents and fails to form maltol in alkaline solution. The hydrogenation does not cause any loss of activity. Treatment of streptomycin B and its dihydro derivative with dilute acids liberates streptidine (or possibly an isomer thereof) isolated as the sulfate monohydrate. Analysis: calculated for $C_8H_{18}O_4N_6 \cdot H_2SO_4 \cdot H_2O$, C 25.40, H 5.86, N 22.20, S 8.46; found, C 25.42, H 5.73, N 22.14, S 8.33. The presence of the maltol-forming moiety, streptose, and of a hexosamine moiety in streptomycin B appears probable from the above data.

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[4] Donovick, R., and Rake, G., to be published.

THE USE OF "COUNTER-CURRENT DISTRIBUTION" FOR THE ANALYSIS OF STREPTOMYCIN PREPARATIONS

Sirs:

Examination of streptomycin preparations for homogeneity by the Craig technique of counter-current distribution[1] indicates the existence of other active structurally related substances.

To prepare the two liquid phases required for distribution, p-toluenesulfonic acid was made up to 5 gm. per 100 cc. in water previously saturated
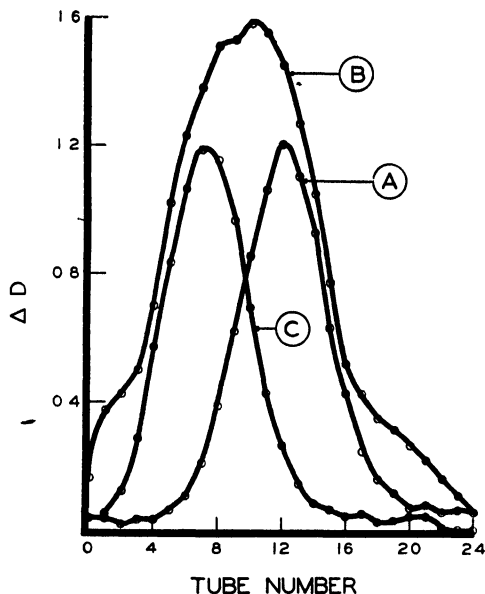
FIG. 1. Distribution curves of, Curve A, a purified preparation of streptomycin; Curve B, a crude streptomycin concentrate; Curve C, streptomycin B reineckate. D indicates the change in optical density at 325 mμ of a solution heated for 10 minutes at pH 12

with butanol. This solution was shaken with an equal volume of butanol previously saturated with water. The partition coefficient of streptomycin between the resulting two layers remained at unity for concentrations ranging from 100 to over 5000 units per cc. This assures agreement between the experimental curves for pure preparations and those calculated from theory.

[1] Craig, L. C., *J. Biol. Chem.*, **155**, 519 (1944). Craig, L. C., Golumbic, C., Mighton, H., and Titus, E., *J. Biol. Chem.*, **161**, 321 (1945).

Streptomycin concentrations were measured in the lower layer by the spectrophotometric determination of the maltol[2] produced by a 10 minute heating at 100° and pH 12. After each twenty-four plate distribution with 8 cc. layers, 80 per cent of the streptomycin was displaced to the lower phase by shaking the contents of each tube with 4 cc. of benzene. The concentrations in the lower layers were then plotted as ordinates against tube numbers.

Curve A of Fig. 1 illustrates the result with a purified preparation of streptomycin. The distribution of a cruder streptomycin (Curve B) revealed fractions occurring in Tubes 5 to 9 and 16 to 22 whose positive Sakaguchi reactions and maltol production indicate their relation to streptomycin. Of these, the former has been isolated,[3] and the distribution of a sample of its reineckate plotted as Curve C. The second substance is under investigation.

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[2] Schenck, J. R., and Spielman, M. A., *J. Am. Chem. Soc*, **67**, 2276 (1945).
[3] Fried, J., and Titus, E., *J. Biol. Chem.*, **168**, 391 (1947).

TYROSINE OXIDATION BY LIVERS FROM RATS WITH A SULFASUXIDINE-INDUCED PTEROYLGLUTAMIC ACID DEFICIENCY

Sirs:

Evidence that tyrosine metabolism is altered in pernicious anemia,[1] and that a pteroylglutamic acid deficiency exists in this disease,[2] suggested an investigation to determine whether pteroylglutamic acid (PGA) *per se* influenced the oxidation of tyrosine.

PGA deficiency was produced in rats by placing them on a purified diet containing sulfasuxidine until leucopenia developed.[3] Livers from the PGA-deficient rats were compared to livers from normal animals as to their effect on tyrosine oxidation in the Warburg apparatus. Livers from three to five animals were pooled and homogenized in a Waring blendor for 20 seconds, with M/15 phosphate buffer, pH 7.5, to give a concentration of 0.25 gm. of wet weight per ml. The oxidation of 0.5 mg. of *l*-tyrosine by 2 ml. of the suspensions was determined over a 2 hour period. A typical experiment is shown in Table I. Average values of three flasks are given.

Addition of sulfasuxidine to normal liver suspension caused only a negligible inhibition of oxygen uptake compared with that of the PGA-deficient liver.

In other experiments 10 γ of crystalline PGA were added to flasks containing liver suspensions from PGA-deficient rats and the effect on the oxidation of tyrosine was determined (Table II).

Under these conditions all experiments showed decreased oxidation of tyrosine by livers from PGA-deficient rats, and a restoration of oxidation on addition of PGA.

Experiments were also carried out with 24 hour-aged suspensions in which the level of oxygen uptake was constant. For a period of an hour the oxygen uptake was determined; tyrosine was then added and the oxygen uptake was measured over a 2 hour period. Here again, livers from PGA-deficient rats showed a decreased oxidation of tyrosine as compared to livers from normal rats in eight of eleven experimental groups. In five of seven experimental groups the rate of tyrosine oxidation by livers from PGA-deficient rats was increased by the addition of PGA.

[1] Swendseid, M. E., Burton, I. F., and Bethell, F. H., *Proc. Soc. Exp. Biol. and Med.*, **52**, 202 (1943).

[2] Moore, C. V, Bierbaum, O., Welch, A. D., and Wright, L. D., *J. Lab. and Clin. Med.*, **30**, 1056 (1945).

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TABLE I

Contents of flask	O_2 used	ΔO_2
	microliters	*microliters*
Liver suspension from normal rats	189 7	
" " " " " + tyrosine	279 0	89 3
" " " PGA-deficient rats	176 2	
" " " " " + tyrosine ..	203 1	26 9

TABLE II

Contents of flask	O_2 used	ΔO_2
	microliters	*microliters*
Liver suspension from PGA-deficient rats	142.7	
" " " " " + tyrosine ..	167 5	24 8
Liver suspension from PGA-deficient rats + PGA ..	136 9	
Liver suspension from PGA-deficient rats +PGA + tyrosine	182 8	45 9

From these results it is indicated that pteroylglutamic acid influences tyrosine oxidation.

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SYNTHESIS OF HIPPURIC ACID IN LIVER HOMOGENATE*

Sirs:

The synthesis of hippuric acid from benzoic acid and glycine resembles in several respects the synthesis of a peptide bond. A CONH group is formed, it is α to a carboxyl group, and the free energy of its formation is of the same order of magnitude. The synthesis was demonstrated in liver and kidney slices of a number of animals; it is inhibited by 0.001 M KCN, which is in accord with the view that the necessary free energy is derived from an oxidation.[1]

It has not been possible with tissue slices to elucidate further the energy-coupling reaction. The cell walls evidently retarded or blocked entry into the cells of possible free energy donors. Consequently we have sought experimental conditions which permit the reaction to proceed in tissue extracts in which the cells were disrupted.

We have now obtained synthesis of hippuric acid from benzoic acid and glycine in homogenized guinea pig liver suspended in a phosphate-saline solution. To obtain more than traces of synthesis it was necessary to homogenize the liver in the presence of the substrate, *i.e.* of benzoic acid and glycine. The yield of hippuric acid was nearly doubled when adenylic acid and α-ketoglutaric acid were added. Evidently the oxidation of the latter substance provided ATP[2] which in turn furnished the free energy for the synthesis. This surmise is based on experiments on the methylation of guanidoacetic acid by methionine, a reaction which requires ATP.[3]

The reaction mixture contained in 500 ml. 10 gm. of homogenized guinea pig liver, 0.02 M glycine, 0.01 M benzoic acid, 0.001 M adenylic acid, and 0.01 M α-ketoglutaric acid in a phosphate-saline solution.[4] The reaction was carried out under oxygen at 38°. The same results were obtained at pH 7.0 and 7.5. Hippuric acid was determined by isolation and identified by crystal form, melting point, and elementary analysis.

Some characteristic findings appear in the table.

Thermodynamic data give the equilibrium constant for the hydrolysis of hippuric acid under our experimental conditions as 211;[1] accordingly the amount of hippuric acid to be expected from 0.02 M glycine and 0.01 M benzoic acid by mass action alone is 0.026 mg. The isolation of 30 mg.

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[4] Cohen, P. P., and Hayano, M., *J. Biol. Chem.*, **166**, 251 (1946).

	Hippuric acid isolated
	mg.
Liver alone	0
" homogenized first and complete reaction mixture added subsequently	0–3
Liver homogenized with benzoic acid and glycine	17
" " " " " glycine, adenylic acid, and α-ketoglutaric acid, 0 time	0
Liver homogenized with benzoic acid, glycine, adenylic acid, and α-ketoglutaric acid, 4 hrs. incubation	30

indicates that the synthesis had occurred through coupling with an energy-yielding reaction, presumably ATP. Under our experimental conditions the isolation is not quantitative; at least 10 mg. are not recovered.

Experiments on the methylation of guanidoacetic acid indicated that guinea pig liver homogenate provides some ATP. This probably accounts for the yield of 17 mg. of hippuric acid when no adenylic acid and α-ketoglutaric acid were added to the reaction mixture.

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INHIBITION OF OXIDATION OF SUCCINIC ACID BY STRUCTURALLY RELATED SULFONIC ACIDS

Sirs:

In their original work on the inhibition of the succinoxidase system by structural analogues of succinic acid, Quastel and Wooldridge[1] have suggested that the formation of an enzyme-inhibitor complex requires the presence of a —C—CH—COOH group "associated possibly with another carboxyl group." It has been pointed out by Potter and Elvehjem[2] that the —CH— group is not essential since oxalic acid also possesses inhibitory properties. It has seemed of interest, therefore, to determine whether the carboxyl groups also can be replaced, *e.g.* by sulfonates, particularly since these groups compete in the formation of complexes with serum albumin,[3] as well as in certain metabolic reactions in bacterial systems.[4]

Investigations have been carried out, therefore, on the inhibition of the succinoxidase system by β-sulfopropionic acid, HO_3S—CH_2—CH_2—$COOH$, and by 1,2-ethanedisulfonic acid, HO_3S—CH_2—CH_2—SO_3H. The procedure was essentially the same as that of Potter and Elvehjem.[2, 5] The results are summarized in the table.

Oxygen Uptake (Microliters per Hour) of Rat Liver Homogenates

The main compartments of Warburg flasks contained 0 4 ml of buffered rat liver homogenate, 0 1 ml. of $M/15$ phosphate buffer (pH 7.4), varying amounts of inhibitor (in the form of its sodium salt), and sufficient water to make 2 ml The side arms contained 0.1 ml of sodium succinate, 0.25 ml. of buffer, and 0 65 ml. of water. The center cups contained 0.2 ml. of 20 per cent KOH. The final concentration of the succinate was 0.02 M.

Inhibitor	None	0.0033 M	0 0067 M	0.0133 M	0.020 M
1,2-Ethanedisulfonic acid	204	150	140	95	73
β-Sulfopropionic acid	127	106	93	62	40

From the results obtained it is evident that these sulfonic acids produce pronounced inhibitions of the succinoxidase system. In fact, the inhibitory activity is almost as great as that of malonic acid. Apparently the sulfonate ion is capable of forming an enzyme complex of almost the same

[1] Quastel, J. H., and Wooldridge, W. R., *Biochem. J.*, **22**, 689 (1928).
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strength as that with malonate. Approximately equal affinities have been observed also in binding by serum albumin.[3]

Quantitative kinetic measurements are in progress to determine whether the inhibition is truly of the competitive type.

The authors wish to express their appreciation to Professor M. S. Kharasch of the University of Chicago and to Professor S. M. McElvain of the University of Wisconsin for furnishing the β-sulfopropionic acid and 1,2-ethanedisulfonic acid, respectively.

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STREPTOMYCIN. THE LINKAGE BETWEEN STREPTIDINE AND STREPTOBIOSAMINE

Sirs:

Recent investigations conducted in several different laboratories have shown that streptomycin is composed of a base, streptidine, linked glycosidically to a nitrogen-containing disaccharide, streptobiosamine. Streptidine has been shown to be one of the eight meso forms of 1,3-diguanido-2,4,5,6-tetrahydroxycyclohexane. Streptobiosamine is composed of N-methyl-L-glucosamine and streptose. In the streptomycin molecule streptidine is linked to the 1st carbon atom of streptose but the point of attachment of streptobiosamine to streptidine (2, 4 (6), or 5) remains to be elucidated.
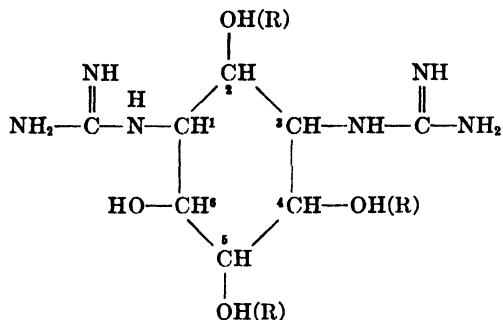
Results obtained in a study of the periodate oxidation of streptidine and of streptomycin have furnished information on this point. Streptidine consumed 2 moles of periodate. During the course of the reaction the positive Sakaguchi test (due to the guanidine groups in streptidine) disappeared and a positive Pauly test appeared. The latter presumably is due to an imidazole derivative formed by ring closure of the guanido-glutaraldehyde resulting from periodate oxidation of streptidine (1-guanido-2,3-dihydroxypropane behaved similarly). In marked contrast to these results, streptomycin solutions after treatment with an excess of periodate at pH 5 to 6 for 72 hours gave a positive Sakaguchi test and a negative Pauly test.

From the streptomycin-periodate oxidation mixture was isolated a degradation product (I) which decomposes at 265°, has a rotation of +74°, and is extremely labile toward acids. Acid treatment of I yields streptidine, glyoxal, and an unidentified acid.

It is apparent, therefore, that streptobiosamine is attached to streptidine in such a position as to interfere with oxidation of the streptidine ring by

periodate. Linkage at position 5 satisfies this condition, since it eliminates the 1,2-glycol grouping needed for periodate action. Position 2 can be eliminated as a possibility. Position 4 cannot be entirely disregarded since in one instance (1,2,3,4-tetraacetylinositol) a 1,2-glycol is reported to be resistant to periodate oxidation.[1]

This work is being continued, but the data already available strongly indicate that in the streptomycin molecule streptobiosamine is attached to streptidine at carbon 5.

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THE ENZYMIC SYNTHESIS OF GLUTAMINE*

Sirs:

An investigation of the enzymic synthesis of glutamine was undertaken as a step toward the study of the synthesis of peptide bonds. From the results of Krebs and coworkers,[1] it seemed likely that the coupling of glutamate and ammonia is achieved with energy derived from adenosine triphosphate, and the present work supports this view.

Glutamine is formed rapidly from glutamate and ammonia by isotonic Waring blendor dispersions of fresh pigeon liver. The greatest activity

Glutamine Synthesis in Pigeon Liver Extracts

The enzyme was prepared by extracting 2 gm of pigeon liver acetone powder with 18 ml. of 0.15 M NaCl, 0 02 M NaHCO₃, 0 001 M cysteine, and dialyzing 16 hours against the same medium at 0°, with internal and external mixing The samples contained 0 03 M NaHCO₃, 0 003 M MgSO₄, 0 04 M sodium $l(+)$-glutamate, NH₄Cl or neutralized NH₂OH·HCl, 0 0045 M adenosine triphosphate, 0 01 M cysteine, and 0 15 ml of extract, in a volume of 1 0 ml. Gas 5 per cent CO₂-95 per cent N₂; temperature 38°; time 10 minutes Hydroxamic acid is expressed in terms of a standard prepared from succinic anhydride.

System	With 0 01 M NH₄Cl		With 0.3 M NH₂OH
	Amide formed	Inorganic phosphate formed	Hydroxamic acid formed
	micromoles	*micromoles*	*micromoles*
Complete	1.91	2.46	1.41
Without adenosine triphosphate	0.08		0.14
" glutamate	0.13	0.54	0.11
" NH₄Cl	0 44	0.70	
" MgSO₄	0.42	0.50	0.14
" cysteine	0.80	1 34	0.97
With 0.001 M NaF	0.86	1.28	0 68

was found in systems which contained, in addition to the two substrates, oxygen in the gas phase, cytochrome c, diphosphopyridine nucleotide, oxalacetate or citrate, phosphate, potassium, and magnesium. No acceleration by adenosine triphosphate was observed.

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A clearer picture of the synthesis of glutamine was obtained by using extracts of acetone-dried pigeon liver as the source of the enzyme system. Glutamine is formed when glutamate, ammonia, adenosine triphosphate, and magnesium ions are added to such an extract. The reaction is more rapid under nitrogen than in air and is accelerated by the addition of cysteine. Liberation of inorganic phosphate from adenosine triphosphate occurs at a rate paralleling the rate of glutamine synthesis. Both glutamine formation and liberation of inorganic phosphate are inhibited by fluoride in low concentrations. Hydroxylamine at levels of 0.01 to 0.3 M may be substituted for ammonia, and the product of the reaction can be determined as hydroxamic acid by the method of Lipmann and Tuttle.[2] Data from a typical experiment are given in the accompanying table.

These results suggest that a reaction occurs between glutamate, ammonia (or hydroxylamine), and adenosine triphosphate, to form the amide, inorganic phosphate, and adenosine diphosphate or monophosphate. The enzyme system appears also to include Mg^{++} as a cofactor and to involve proteins requiring sulfhydryl groups for full activity. The system for glutamine synthesis is quite similar to those involved in the acetylation of choline[3] and of sulfanilamide.[4]

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CORRECTIONS

On page 112, Vol. 167, No. 1, January, 1947, line 5 of the small type, read *21.71*. for *21.62*.

On page 201, Vol. 167, No. 1, January, 1947, ordinate scale of Fig. 1, read $C_{substrate}$ $(k_1/mg.\ enzyme\ N) \times 10^3$ for $C_{substrate}\ (k_1/mg.\ enzyme\ N)$.

On page 331, Vol. 167, No. 2, February, 1947, line 13, read *casein* for *crystalline casein*.
On page 335, Table IV, column 1, read *casein* for *crystalline casein*.

On page 737, Vol. 167, No. 3, March, 1947, throughout the article read *N-(4((4-quinazoline)amino)benzoyl)glutamic acid* for *N-(4(4-quinazoline)benzoyl)glutamic acid*.

ENDOCRINE REGULATION OF AMINO ACID LEVELS IN BLOOD AND TISSUES

By FELIX FRIEDBERG and DAVID M. GREENBERG

(From the Division of Biochemistry, University of California Medical School, Berkeley)

Evidence has accumulated which indicates that hormones play an important rôle in protein metabolism. For example, according to the current concept growth hormone stimulates protein anabolism (1) and adrenal cortical extract increases glucose neogenesis at the expense of protein (2). Various investigators have tried to relate changes in the free amino acid of the blood to the endocrine regulation of protein metabolism. The reports of previous workers have not been in complete · agreement, probably because of differences in analytical methods employed, variations in endocrine extract, dosage, the route of administration, and time relationships.

According to the majority of papers, insulin causes a lowering of the amino acid level (3–5), and Greene's claim (6) that insulin had no effect has not been supported. Okada (7) reported that epinephrine does not change the amino acid concentration of rabbit blood. Luck and coworkers, however, state that epinephrine alone is as effective as insulin in producing hypoaminoacidemia (8, 9), while still another group of investigators found an increase (10). A drop in the plasma amino acid level was shown to occur with hypophysectomy and with thyroidectomy (11), but Okada (7), who could not obtain any change when epinephrine was given, also claims that neither the removal of the thyroid gland nor hyperthyroidism influences the amino nitrogen content of blood. Investigations of the action of thiouracil treatment, adrenalectomy, estrogen administration, and prolonged adrenocortical steroid injections have been neglected.

The present project was started to clarify earlier work on the influence of endocrines on amino acid metabolism and was extended to include not only plasma, but also liver, kidney, and skeletal muscle. The gasometric ninhydrin reaction (12) was chosen in preference to the colorimetric procedure (13), the gasometric nitrous acid method (14), and the formaldehyde titration (15) because of its specificity for free amino acids.

EXPERIMENTAL

The animals were anesthetized with nembutal. Blood was drawn from the heart, heparin being used as anticoagulant. 2 ml. of plasma were

treated with 10 ml. of 1 per cent picric acid and analyzed for amino acids by the ninhydrin-carbon dioxide method of Hamilton and Van Slyke (12). About 1 gm. of tissue, previously frozen in dry ice and acetone, was ground with powdered glass and then extracted with two 10 ml. portions of 1 per cent picric acid. After centrifugation and filtration, 5 ml. of the filtrate were analyzed in the same manner as the plasma filtrates. If necessary, the tissue can be stored after lyophilization to a dry powder. The powder is extracted in a Waring blendor and the analysis is the same as for frozen tissue; however, the weight of the water removed must be recorded.

Hypothyroidism—This condition was caused by adding 0.2 per cent thiouracil (deracil, Lederle Laboratories, Inc.) to the diet for 6 weeks. At sacrifice the rats were examined for enlargement of the thyroid.

Thyroidectomy—A period of 6 weeks elapsed after the operation.

Hyperthyroidism—This condition was produced by feeding 1 per cent thyroid powder (Armour Laboratories) in the diet for 3 weeks. Only animals that lost weight during the treatment were used.

Adrenalectomy—During the postoperative period, 1 per cent sodium chloride was administered in order to sustain life and prevent hemoconcentration   10 days after the operation the food was removed, the salt water was replaced by ordinary water, and, as soon as adrenal crisis set in (muscular weakness, etc.), the animal was sacrificed.

Lipo-Adrenal Cortex Extract—Three injections of 2 ml. of lipo-adrenal cortex[1] (80 rat units) at 2 day intervals were administered subcutaneously.

Epinephrine—1.5 mg. per kilo of body weight were given subcutaneously to rats fasted 12 hours. The animals were sacrificed after 1 hour.

Insulin—20 units per kilo of body weight were injected subcutaneously into rats fasted 12 hours. The animals were sacrificed after 3 hours.

Hormone Treatment—*Estrogen*, 500 rat units (progynon-B),[2] was administered subcutaneously each 3 days for 4 weeks.

Hypophysectomy—The animals were hypophysectomized when 40 days old and sacrificed 18 days later. Only animals that showed a definite loss in weight were used.

Non-Protein Diet—The diet consisted of 54.7 per cent sucrose, 25 per cent starch, 15 per cent Crisco, 5.3 per cent salts, and vitamins. The rats on this regimen were fed for 8 weeks, when they had lost about one-third of their weight.

In all experiments, normal male rats weighing between 100 and 200 gm. were used. They were fasted 12 hours before sacrifice.

The data obtained are given in Table I.

[1] Generously donated by The Upjohn Company through the kindness of Dr. D. J. Ingle.

[2] Generously donated by the Schering Corporation through the kindness of Dr. W. H. Stoner, Medical Director.

DISCUSSION

Analysis of amino acid levels is made possible by the constancy of the normal concentration in blood and tissues. Each tissue has its own characteristic value, which is about 3 to 9 times that of blood. It is not known how the amino acids are concentrated by the tissues. Our values for rat liver are slightly lower and the values for skeletal muscle are less than

TABLE I

Free Amino Acid Levels in Blood and Tissues

Treatment	Amino nitrogen			
	Plasma	Liver	Kidney	Skeletal muscle
	mg per 100 ml.	*mg. per 100 gm.*	*mg. per 100 gm.*	*mg. per 100 gm.*
Normal	(7) 6.4 ± 0.1	(7) 32.1 ± 1.7	(7) 44.0 ± 1.7	(7) 19.2 ± 1.4
Thiouracil	(5) 5.2 ± 0.2	(5) 32.3 ± 1.5	(5) 42.6 ± 1.0	(5) 20.4 ± 0.9
	<0.01*	>0.05	>0.05	>0.05
Thyroidectomy	(7) 5.0 ± 0.3	(7) 33.0 ± 1.6	(7) 40.2 ± 0.9	(7) 20.1 ± 1.3
	<0.01	>0.05	<0.05	>0.05
Thyroxine	(4) 9.0 ± 0.6	(4) 44.0 ± 3.6	(4) 42.3 ± 2.1	(4) 28.1 ± 3.3
	<0.01	<0.01	>0.05	<0.01
Adrenalectomy	(6) 5.2 ± 0.2	(5) 31 0 ± 2.2	(5) 44.6 ± 2.8	(5) 27 0 ± 1.8
	<0.01	>0 05	>0.05	<0.01
Adrenal cortex	(5) 7.9 ± 0.6	(5) 41.5 ± 1.7	(5) 45.7 ± 3.6	(5) 24.4 ± 1.0
	<0.01	<0.01	>0.05	<0.02
Epinephrine	(4) 4.5 ± 0.4	(4) 35.7 ± 1.4	(4) 33.1 ± 3.3	(4) 20.7 ± 1.7
	<0.01	>0.05	<0.01	>0.05
Insulin	(5) 4.8 ± 0.6	(5) 28.0 ± 1.3	(5) 34.5 ± 1.5	(5) 18.5 ± 1.5
	<0.01	>0.05	<0.01	>0.05
Estrogen	(6) 5.2 ± 0.4	(6) 31.4 ± 1.3	(6) 39.0 ± 1.2	(6) 22.5 ± 1.7
	<0.02	>0.05	<0.02	>0.05
Hypophysectomy	(6) 5.7 ± 0.2	(6) 29.2 ± 1.8	(6) 31.9 ± 1.7	(6) 20.7 ± 1.5
	<0.01	>0.05	<0.01	>0.05
Non-protein diet	(4) 10.8 ± 0.6	(4) 53.4 ± 2.6	(4) 49.4 ± 3.1	(4) 22.1 ± 2.5
	<0.01	<0.01	>0.05	>0.05

The figures in parentheses represent the number of animals.

The values represent the arithmetic mean plus or minus the standard error of the mean.

* Probability (if P values are 0.05 and less the result is significant); Fisher (16).

half of those given by Luck (17). The amino acid levels obtained on dogs by Hamilton (18), using the ninhydrin reaction, show great variations. Hence, they cannot readily be compared, but they are lower than those found by Van Slyke (19) with the nitrous acid method. The highest amino acid concentrations were observed by us in the kidney and the lowest in the skeletal muscle (Tables I and II). The most constant concentration was found in blood plasmas.

Every hormone so far tested affects the amino acids of blood and tissues. The metabolism of carbohydrate, protein, and fat is interrelated. The action of hormones on the amino acids is probably indirect and due to primary changes of other metabolites. In addition there may be tropic effects between different endocrines. Insulin, epinephrine, estrogen, hypophysectomy, thiouracil treatment, and thyroidectomy, decrease the blood amino acid concentration; thyroxine, lipo-adrenal cortex extract, and also protein starvation increase it. In the kidney, insulin, epinephrine, estrogen, and hypophysectomy produce a significant fall. Thyroxine, lipo-adrenal cortex extract, and protein starvation elevate the liver amino acid level. Thyroxine and lipo-adrenal cortex extract also bring about an increase of the free amino acids in muscle. Adrenalectomy results in hypoaminoacidemia but simultaneously causes a rise in the free amino acid level of muscle.

TABLE II

Free Amino Acid Levels for Few Normal Tissues

Tissue	Amino nitrogen per 100 gm.
	mg.
Brain	(4) 43.1 ± 1.8
Testis	(2) 26.7 ± 0.9
Spleen	(2) 36.0 ± 2.7
Thoracic lymph	6.7*

The figures in parentheses represent the number of animals.

The values represent the arithmetic mean plus or minus the standard error of the mean. .

* Amino nitrogen per 100 ml of lymph obtained by pooling.

It is known that muscular wasting occurs in adrenal insufficiency which may be overcome by epinephrine injections (20).

The changes in amino acids are most definite in the blood and are less marked in the tissues. A lowered level will show up more readily in kidney than in liver, while an increased level can be detected better in liver than in kidney.

Interpretation of the amino acid changes should be delayed until more direct evidence on their metabolism is at hand. A decreased amino acid level could mean (1) decreased proteolysis of tissue proteins, (2) increased utilization of amino acids for protein synthesis, glycogenesis, lipogenesis, and energy production, or (3) increased elimination.

In animals on a non-protein diet, the plasma amino acid level is approximately doubled, the liver level is raised considerably, while the muscle and kidney values increase only slightly. This is in contrast to the findings of Goettsch *et al.* (21) who state: "It was rather a surprise to find that the

value of plasma amino acid during fasting was maintained at a normal level in spite of the severe degree of muscle wasting which occurred as hypoproteinemia progressed " Endocrine disturbances often result in weight changes, which in turn might affect the amino acid levels.

SUMMARY

Insulin, epinephrine, estrogen, hypophysectomy, thiouracil treatment, and thyroidectomy decrease the blood amino acid concentration; thyroxine, lipo-adrenal cortex extract, and also protein starvation increase it.

Adrenalectomy results in hypoaminoacidemia but simultaneously causes a rise in the free amino acid level of muscle.

In the kidney, insulin, epinephrine, estrogen, and hypophysectomy produce a significant fall. Thyroxine, lipo-adrenal cortex extract, and protein starvation elevate the liver amino acid level. Thyroxine and lipo-adrenal cortex extract also bring about an increase of the free amino acids in muscle.

The amino acid concentration is highest in kidney and brain and decreases in other tissues in the following order, spleen, liver, testis, muscle.

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PARTITION OF INTRAVENOUSLY ADMINISTERED AMINO ACIDS IN BLOOD AND TISSUES

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The increasing use of protein hydrolysates in clinical medicine has aroused renewed interest in the utilization and fate of parenterally administered amino acids and amino acid mixtures in the body. The levels of the amino acids of the tissues are from 3 to 9 times greater than in the blood plasma. How the tissues maintain their concentration differential is not understood. The concentrations of the amino acids of the blood plasma and tissues are extremely constant normally and are but little affected by physiological changes or most pathological conditions.

The present investigation was undertaken to determine whether all amino acids are concentrated to the same degree in representative tissues of the body when introduced into the animal in relatively large amounts by intravenous injection.

The tests were carried out with solutions containing equivalent amounts of amino nitrogen of a protein hydrolysate (parenamine, Stearns), and the amino acids glycine, *l*-alanine, *l*-glutamic acid, *l*-histidine, and *l*-lysine.

EXPERIMENTAL

The chemical methods for most of the amino acids are not of sufficient specificity, accuracy, or simplicity to be employed in following changes in the concentrations of these substances in blood plasma and tissues. The gasometric ninhydrin reaction (1) is an analytical method for determining all amino acids, but changes in the level of an individual amino acid may be assayed by this reaction if a large dose is injected and the subsequent rise in amino nitrogen is significant enough to be attributed to the amino acid in question. This procedure was employed in our work. The determinations were carried out on plasma and tissues as described in another publication (2).

For administration, the amino acids were dissolved in 10 ml. of 0.9 per cent sodium chloride and the solution was adjusted to pH 7.4 with sodium bicarbonate. Doses of 0.16 gm. of amino nitrogen per kilo of body weight were injected by way of the jugular vein. Male rats weighing between 100 and 200 gm., which had been fasted for 12 hours, were used. The animals were sacrificed 15 minutes after the injection; blood plasma

and the desired tissues were collected and picric acid filtrates prepared for analysis of the amino acids (2). The analytical data obtained are recorded in Table I.

TABLE I

Free Amino Acid Levels in Plasma and Tissues 15 Minutes after Intravenous Administration of Amino Acids*

Substance administered	Plasma amino N	Per cent adminis- tered amino N removed from plasma†	Amino N per 100 gm. fresh weight			
			Liver	Kidney	Skeletal muscle	Brain
	mg. per 100 ml		*mg.*	*mg.*	*mg.*	*mg.*
Normal values	6.4 ± 0.1		32.1 ± 1.7	44.6 ± 1.7	19.2 ± 1.4	43.1 ± 1.8
Protein hydroly- sate‡	29.4 ± 1.4	87 ± 0.8	61.7 ± 4.7	81.1 ± 3.7	34.1 ± 4.5	43.9 ± 1.5
Glycine	29.7 ± 2.8	87 ± 1.6	74.4 ± 8.3	76.8 ± 2.6	35.2 ± 3.5	42.4 ± 1.6
l-Glutamate (mono- sodium)	43.6 ± 6.2	80 ± 3.3	39.6 ± 3.2	94 3 ± 2.9	28.8 ± 0.7	42.1 ± 1.5
l-Histidine (monohy- drochlo- ride)	26.6 ± 3.5	89 ± 1.9	50.7 ± 4.7	70.2 ± 2.5	29.1 ± 2.9	43.2 ± 3.1
l-Lysine (di- hydrochlo- ride)	28.5 ± 3.0	88 ± 1.4	62.4 ± 2.9	100.6 ± 6.5	32.7 ± 2.9	43.0 ± 1.3
l-Alanine	25 1 ± 2.2	90 ± 0.9	76.6 ± 4.6	62.2 ± 4.1	30.7 ± 0.9	41.9 ± 1.2

* The data represent the arithmetic mean plus or minus the standard error of the mean of groups of four animals in each series except for the normal values, which are for seven animals.

† Calculated on the assumption that the blood is 8 5 per cent of the body weight and that the normal plasma amino acid level is 6.0 mg. of amino nitrogen per 100 ml.

‡Generously donated by Frederick Stearns and Company through the kindness of Dr. M. Sahyun.

DISCUSSION

The amino acids were found to be removed from the b ood plasma to the extent of 80 to 90 per cent with.n 15 minutes after intravenous adminis- tration. The amino acid most slowly cleared was *l*-glutamic acid. Liver and kidney were observed to be most active in concentrating the injected amino acids, skeletal muscle less so, and brain showed no increase what- soever. This behavior of brain is interesting because, in spite of this apparent barrier to the uptake of amino acids, the normal value of free amino acids in brain tissue is 7 times as great as that in blood plasma.

While there was an increase in the amino acid content of all of the representative tissues tested except brain, the pattern of concentration was not the same for all the amino acids examined. 15 minutes after injection high amino acid values were found in kidney and skeletal muscle with *l*-lysine. *l*-Histidine gave only medium liver and medium (or low) kidney concentrations. Glycine accumulated in skeletal muscle as was previously reported by Luck (3). *l*-Alanine behaved much as did glycine, but it did not exhibit the affinity of that amino acid for skeletal muscle. *l*-Glutamic acid was poorly concentrated in the liver but markedly in the kidney. The protein hydrolysate, as would be expected from its composite nature, was taken up well by all of the tissues except brain.

The findings on glutamic acid and glycine may be correlated with the observation of Madden *et al.* (4) that glutamic acid is poorly and glycine well tolerated when given intravenously. They also harmonize well with the special physiological relationships of glycine to creatine in muscle and glutamic acid to glutamine in the kidney (5).

The variations in the amino acid uptake of the tissues and the mechanism of their concentration must remain unexplained at this time but the observations offer a challenge to further research. It would be of interest to repeat the experiments with analytical methods specific for the individual amino acids, *e.g.* by microbiological assay.

SUMMARY

Intravenously administered amino acids are rapidly removed from the blood plasma. They are highly concentrated by liver and kidney, less so by skeletal muscle, and not at all by brain. The pattern of concentration is not the same for all of the amino acids examined.

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A STATISTICAL EVALUATION OF THE THIAMINE AND PYRAMIN* EXCRETIONS OF NORMAL YOUNG MEN ON CONTROLLED INTAKES OF THIAMINE†

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The urinary excretion of thiamine has been used for some time as an empirical indication of thiamine nutrition. The tacit assumption is made that when the dietary intake of the vitamin is at or below the human "requirement" the urinary excretion falls below a certain value, but as long as the dietary intake exceeds the requirement the urinary excretion remains above this critical level (10).

This assumption was originally made without a clear understanding of the range of the variation in excretion values under controlled dietary conditions. In most cases the controlled studies were made over very short periods, so it is likely that many of the subjects had not attained equilibrium in their urinary excretion of thiamine. Furthermore, in many cases, the methods used for the determination of thiamine in the urine are open to some question (12).

Very few reports have utilized statistical methods, even where the data were sufficient to warrant such use. A notable example of this is the series of papers by the group at the Mayo Clinic (9, 18). Although they collected urine samples from subjects who were maintained on constant thiamine intakes for periods as long as 7 months, only a single average value for the urinary thiamine is given for each period. Elsom, Reinhold, Nicholson, and Chornock (4) have given the standard deviations for the urinary thiamine excretions of nine women who consumed a constant amount of thiamine for indefinite periods. They and Wertz and Mitchell (17) are the only investigators who seem to have given this problem any considera-

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tion, but their data are so scanty that a true picture of the variation in urinary thiamine excretion is not possible. Their results, however, do bring out the great variation in the urinary excretion of thiamine by "normal" adults.

The present paper presents a statistical evaluation both of thiamine and of pyramin excretions by normal young men of comparable body size who were living under the same conditions of diet and activity. These experiments have been carried on in this Laboratory from 1943 to date. They have involved the prolonged maintenance of the subjects on controlled thiamine intakes from zero up to 16 mg. per day. A survey of the general results of one of these experiments has already been published (8).

EXPERIMENTAL

In one experiment, groups of two, four, and four men were given 1.81, 1.01, and 0.61 mg. of thiamine per day, respectively, for a period of 24 weeks. In another experiment, two groups of six subjects each received 1.00 and 2.00 mg. of thiamine, respectively, per day for a period of 34 weeks, after a control period of a month on the lower level of thiamine intake. Following this experiment the intake levels of the two groups were reversed. A period of 1 month on a partially controlled diet intervened between the two parts of the latter experiment. In each of these experiments, all men received the same basic diet. All meals throughout the experiment were served as weighed portions from the diet kitchen. The differences in the thiamine intake were adjusted by means of thiamine pills and placebos. The thiamine content of the diet averaged close to 0.5 mg. per day. The day to day variation of the dietary intake of thiamine from the given mean values was of the order, $\sigma = \pm 0.06$ to 0.08 mg. per day. This was determined by actual analyses of the diets served. Finally, the excretion values at higher levels of intake, *i.e.* 5, 11, and 16 mg. of thiamine per day, were obtained from other subjects on somewhat less strictly controlled thiamine intakes.

Analytical Methods

In all of these experiments, except those in which 5, 11, and 16 mg. of thiamine were used, diet samples were collected daily. An exact duplicate of all the food served to the subjects was saved for analysis. This was analyzed for thiamine by the method of Hennessy and Cerecedo (6).

All of the values reported in this paper for urinary thiamine excretion represent 24 hour samples which were collected in bottles containing 5 ml. of toluene and 5 ml. of glacial acetic acid as preservatives. Thiamine was determined in these samples by our modification of the thiochrome procedure (12).

The pyramin content of the urine was determined by a modification of the yeast fermentation method of Schultz, Atkin, and Frey (15). According to the original description of the method, the pyramin value is represented by the sulfite blank. The standard used in these determinations was 2-methyl-4-amino-5-ethoxymethylpyrimidine hydrochloride.

Statistics

Many of the statistical methods used in the analysis of our results are not available to most biochemists or physiologists. To clarify our methods and to avoid confusion as to the definition of terms, we include a brief summary of the statistical items used in this report. For a more complete discussion and for the mathematical derivation of the formulas, see Snedecor (16).

The variability of the thiamine and pyramin excretion data has been submitted to the analysis of variance (16). In the discussion of variability, the variances σ_{id}^2, σ_i^2, σ_d^2, σ_{wi}^2, and σ_{wd}^2 are related to the mean squares, V_{td}, V_i, V_d, V_{wd}, and V_{wi}, as indicated in the following equations:

$$(1) \qquad V_{wi} = \sigma_{wi}^2 = \sigma_{id}^2 + \sigma_d^2 = \frac{\Sigma y^2 - \Sigma I^2/k}{nk - n}$$

$$(2) \qquad V_{wd} = \sigma_{wd}^2 = \sigma_{id}^2 + \sigma_i^2 = \frac{\Sigma y^2 - \Sigma D^2/n}{nk - k}$$

$$(3) \qquad V_i = \sigma_{id}^2 + k\sigma_i^2 = \frac{\Sigma I^2/k - T^2/nk}{n - 1}$$

$$(4) \qquad V_d = \sigma_{id}^2 + n\sigma_d^2 = \frac{\Sigma D^2/n - T^2/nk}{k - 1}$$

$$(5) \qquad V_{id} = \sigma_{id}^2 = \frac{\Sigma y^2 - \Sigma D^2/n - \Sigma I^2/k + T^2/nk}{nk - k - n + 1}$$

where, in a given table of data showing the excretion values, y, for n individuals on k days, the sums of the excretion values for each of the individuals over this period of days are $I_1, I_2, I_3,$ I_n, respectively; the daily totals summing all individual values for each day are $D_1, D_2, D_3, \ldots, D_k$, respectively, and T is the grand total of all nk values of y.

These terms are measures of:

$V_{wi} = \sigma_{wi}^2$, the within individual (intraindividual) variation. This is a pooled ("statistically averaged") value for day to day variations for each of the individuals within the group and represents the average day to day variations of each individual from his own mean.

$V_{wd} = \sigma_{wd}^2$, the within day (interindividual) variation. This is a pooled value for the k days of the deviation of individual values on a given day from the mean group value on that day.

V_i, the deviation of the n individual mean values from the grand mean for the total period.

V_d, the deviation of the k daily mean values from the grand mean.

$V_{id} = \sigma^2_{id}$, the interaction term or purely random "error." This can be represented graphically as the degree to which the individual values from day to day fail to move in a parallel fashion.

σ_i, that part of the interindividual variation (not attributable to random error) which is consistent for the individual from day to day.

σ_d, that part of the day to day "general up and down movement" of the group values which is over and above that expected from purely random "error."

TABLE I

Analysis of Variances of 24 Hour Urinary Excretion of Pyramin by Normal Young Men on Different Levels of Thiamine Intake

The men were maintained at each level of intake for periods of 2 or more months (see Table III for the exact time). The urinary excretion values are expressed as micrograms of 2-methyl-4-amino-5-ethoxymethylpyrimidine hydrochloride per day. The thiamine intake is expressed as mg. per day. The definition of σ is given in the text. The pooled values are the weighted averages for each column of figures.

Thiamine intake	24 hr. urinary pyramin excretion				
	Mean	σ_{wd}	σ_{wi}	σ_{id}	σ_i	σ_d
0.61	125.5	21.0	26.3	18.9	8.9	18.2
1.00	165.4	19.0	29.3	18.2	5.3	22.9
1.01	171.5	22.9	30.0	22.0	6.5	20.5
1.81	267.4	27.7	40.1	29.4	0	27.4
2.00	232.6	23.1	28.9	21.5	8.4	19.4
Pooled values ..		21.58	29.65	20.45	6.90	21.48

Excretion of Pyramin—The values of the different variances (Table I) are quite constant for pyramin, being homogeneously distributed throughout the data from all of the experimental groups on all levels of thiamine intake and pyramin excretion.

The pyramin values from all of our pooled experimental data have been used in calculating the following variations in micrograms per day:

$$\sigma_{wi} = 30.46; \; \sigma_{wd} = 22.61; \; \sigma_{id} = 20.81$$

In each case there are 500 to 600 degrees of freedom. By calculation from the above values, $\sigma_i = 8.84$ and $\sigma_d = 22.24$. In the urinary pyramin excretion data, σ_i, the individual variation corrected for random "error," is very small, in some cases almost insignificant. Considering the careful

control of the dietary intake of thiamine, the day to day variation as represented by σ_{wd} is surprisingly large. This may be partly due to the fact that, while the diet is carefully controlled with respect to thiamine, it was not possible to control the intake of the pyrimidines which may be related to thiamine or its decomposition products. Direct analysis of the diets for pyramin has been found to be valueless because of the tendency of compounds that are inactive in the fermentation method to become "active" on feeding (3).

Excretion of Thiamine—The variance values in the thiamine excretion data are not so uniformly distributed as they were in the pyramin data. The variation in the thiamine excretion depends to a large extent upon the magnitude of the mean with which it is associated. This has been noted

<div align="center">TABLE II</div>

Analysis of Variance of 24 Hour Urinary Thiamine Excretion Data of Normal Young Men As Calculated by Two Different Methods (See Table III for ·Details)

A, values calculated from actual excretion data expressed in terms of micrograms of thiamine per day, and B, values calculated from the logarithms of the excretion data, expressed in terms of per cent of the mean thiamine excretion.

Thiamine intake	Excretion mean	A, γ B₁ per day					B, per cent of mean				
		σ_{wd}	σ_{u1}	σ_{1d}	σ_1	σ_d	σ_{1rd}	σ_{u1}	σ_{1d}	σ_1	σ_d
mg per day	*γ per day*										
0 61	5	6	6	3	5	6	184	182	104	115	113
1 01	26	16	13	13	9	1	125	95	25	118	88
1.00	65	25	23	16	20	17	41	96	7	40	95
1.81	195	36	29	27	22	13	22	18	16	13	7
2 00	224	106	81	57	90	58	52	26	16	42	21

to a certain extent by other workers (4) who have preferred to express deviations in thiamine excretion in terms of percentages of the mean. This strong dependence of variance upon the mean can readily be seen in Table II, A. Where the variance is so intimately related to the mean, it is proper to convert the excretion values to logarithms before proceeding with the analysis of variance, and then express the final figures as percentages of the mean ((16) p. 448). This helps somewhat, but, as may be seen in Table II, B, even according to this method the variances are not homogeneously distributed throughout the various intake levels. The logarithmic treatment of the thiamine excretion data overcompensates for the trend seen in the ordinary analysis of variance (Table II, A). The proper method of analysis would involve some intermediary statistical procedure which has not yet been developed. Both of the presently available methods

for the analysis of variance, when applied to the thiamine data, yield results which are markedly different and are inconsistent among themselves (Table II). This does not permit large scale pooling of values or even proper comparison of samples, and it makes strict analysis of variance almost impossible with the thiamine data. It has been found possible to obtain reasonable results for the significance of the changes in thiamine excretion following a drastic change in physical activity or composition of diet (the thiamine intake maintained at the previous constant level) by treating the urinary thiamine values on an individual basis. The values for each individual, under these circumstances, must be compared with his own normal values. In this way, σ_i, one of the large variables, can be eliminated from the calculations.

The very large values found for σ_i constitute one of the more striking phenomena of the thiamine excretion data. The thiamine excretion level is highly characteristic of the individual as well as the intake. A group of men on identical and carefully controlled intakes of thiamine will show very different but individually consistent levels of thiamine excretion. Some of the men may consistently excrete twice as much thiamine as others. At the same time, however, the pyramin excretion level of these same individuals will be much less variable. There are no visible outward signs to indicate a reason for this difference in thiamine excretion (13).

The intraclass (or intragroup) correlation, $r = (\sigma_i^2)/(\sigma_i^2 + \sigma_{id}^2)$, is a measure of the consistency with which the various individuals in a group maintain their relative positions within the group ((16) p. 243). The values for each of the groups are given in Table III for both thiamine and pyramin. In general, the thiamine excretions show highly significant values of r close to 0.7 (0.4 to 0.9), while those for pyramin are about 0.1 (0 to 0.2) and statistically non-significant. In the second of the two experiments previously described, the reversal of intake levels of the two groups of men afforded an opportunity to check the consistency with which the individuals of a group hold their relative positions in that group when the intake levels are reversed. It was found that, when the individuals were ranked with respect to their position in the group both before and after the change in intake, the rank correlations ((16) p. 164) obtained were +0.89 and +0.99, as compared with the intragroup correlation values ((16) p. 243) of +0.85 and +0.97, respectively, found for these groups during the control period of 6 to 8 months. This effect is very important, and has been discussed in greater detail elsewhere (13).

All of the experimental groups do not show as high correlation of thiamine intake and excretion values as the above. The lower values for the intragroup correlations obtained at some of the thiamine intake levels are possibly attributable to the facts that (1) at low levels of thiamine intake,

where excretion values and the attendant variabilities all approach zero, the possibility of distinguishing individuals decreases, and (2), since the levels of thiamine excretion are highly dependent upon the individuals in a group, the intraclass correlations obtained from small groups would be expected to vary considerably.

Time Required for Stabilization—One factor that must be kept in mind in attempts to obtain thiamine and pyramin excretion values characteristic of a given intake of thiamine is the period of time required for an individual to come into metabolic equilibrium with the new level of intake. Fig. 1

TABLE III

Summary of Urinary Excretion of Thiamine and Pyramin by Normal Young Men Maintained on Different Levels of Thiamine Intake for Periods of 2 or More Months

The excretion values are given only for the collections made after the men were stabilized at their intake levels. The excretion of thiamine is given in micrograms of thiamine hydrochloride per 24 hours, whereas that of pyramin is in micrograms of 2-methyl-4-amino-5-ethoxymethylpyrimidine hydrochloride. The definitions of the variances and correlation terms are given in the text. The groups with an asterisk are those whose thiamine intakes were reversed after the 8 months experiment.

Daily thiamine intake		No of subjects	Months on intake	Average thiamine excretion				Average pyramin excretion			
mg.	σ			Mean	σ_{wd}	$\sigma_{u\imath}$	r	Mean	σ_{wd}	$\sigma_{w\imath}$	r
0 61	0.07	4	6	4.6	6 3	6.3	0 38	125 5	21.0	26.3	0.18
1.00	0 08	6	8	65 4	25 1	23 4	0.97	165 4	19.0	29.3	0.08
1 00	0 08	6*	3	57 2				155.8			
1.01	0.07	4	6	26 2	15.9	13 0	0.38	171.5	22 9	30.0	0.08
1.81	0.07	2	6	195 4	35 8	29 5	0.54	267.4	27 7	40.1	0.00
2.00	0 08	6	8	224.0	106.3	81.1	0 85	232.6	23.1	28.9	0.13
2.00	0 08	6*	3	249.3				214.9			
5		6	2	971	175	238		307	57.3	46.5	
11		8	2	1875	865	435		378	43.5	56.3	
16		1	2	1430				385		35.7	

shows the rate of change of thiamine and pyramin excretion levels found in a group of six men when they were changed from a constant intake level of 1.00 mg. of thiamine per day to a constant intake level of 2.00 mg. of thiamine per day. The group subsequently remained on this higher level of intake for a period of about 8 months. The plateau values shown represent the average thiamine and pyramin excretion values obtained over approximately the last 6 months of this period. The ordinates have been arranged so that the two plateau values coincide exactly in Fig. 1.

The thiamine excretion values show a sharp and immediate rise, which,

however, does not reach the levels characteristic of the new intake during the first 30 days. The pyramin values rise more slowly than the thiamine in the first few days. This may possibly account for previous reports that pyramin excretion values are insensitive to changes of, or are entirely independent of, thiamine intake. The smooth least squares curve passing through the data in Fig. 1 is

$$\text{Micrograms pyramin} = 67.2 - 67\ 2e^{-0\ 70t}$$
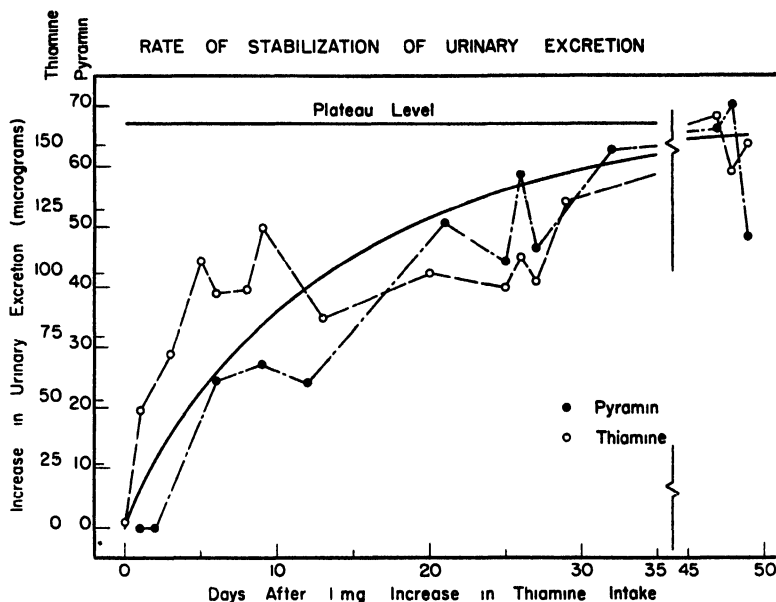
FIG 1 Rate of stabilization of urinary and pyramin excretion when the thiamine intake is changed from 1 to 2 mg per day. The increase in the excretion represents the difference between the two intake groups

where e is the base of the natural system of logarithms, 2.718, and t is the time in days. In this formula $t_{\frac{1}{2}} = 10$ days; that is, this curve traverses half of the remaining distance to the plateau level each 10 days. Multiplication of the values found in the above equation by 2.36 gives the corresponding thiamine values observed in this instance. We have not studied rates of change following a decrease of thiamine intake, nor those encountered at higher or lower levels of thiamine intake. In the normal intake region studied, a group of individuals must be maintained on a carefully controlled thiamine intake for a period of at least 6 weeks before their excretion values may be considered to be truly characteristic of that level of intake.

Because of the great variation in the thiamine excretion data, there is a possibility that certain values may equal or exceed the plateau value before the time indicated in Fig. 1.

Relation between Intake and Excretion—In comparing thiamine and pyramin it must be remembered that the molecular weight of the thiamine standard is almost twice that of the pyramin standard. Thus, on a molar basis, a rise of 60 γ of pyramin is equivalent to a rise of 100 γ of thiamine.

When the average excretion values for both thiamine and pyramin are plotted against the mg. of thiamine ingested per day, the curves in Fig. 2 are obtained. The ever increasing variability in the thiamine data makes it difficult to determine the exact nature of the curve relating these points. There is little to be gained, however, in assuming that it is other than a straight line, especially for excretion values at intakes in excess of 1.0 mg. of thiamine per day. Since each individual shows his own characteristic slope, a great variability must be associated with any value given to the slope of this straight line. Fitting a least squares line to the thiamine excretion data which go to make up the values found in Table II gives the equation

Micrograms thiamine excreted = $-118 + 173 \times$ mg. thiamine intake

This indicates that at intakes below about 0.7 mg. of thiamine per day the urinary thiamine excretion would be zero for most individuals. It must be remembered that the level of urinary thiamine excretion is highly characteristic of the individual; consequently it is rather difficult to make useful generalizations from this.

The literature is in disagreement concerning the thiamine excretions on the lower thiamine intakes. This may be attributed to one or more of the following: (1) individual differences of subjects, (2) inadequate time for stabilization of urinary excretion, (3) difficulties in exactly controlling dietary intakes at these lower levels, (4) differences in analytical methods used in the determination of thiamine, and (5) differences in the physical characteristics, activity, and living conditions of the subjects. The method used for the determination of thiamine is especially important when the concentration of this vitamin in the urine is very low. Mickelsen, Condiff, and Keys (12) have discussed the problems involved under these circumstances.

The relation of the stabilized pyramin excretion values to thiamine intakes (Fig. 2) is an exponential curve which can be represented by the equation

Micrograms pyramin = $400 - 330e^{-0.844}$

where $A = $ daily thiamine intake in mg. The curve approaches a plateau value of 400 γ of pyramin per day for very high intakes of thiamine. The

significance of the change in the slope of the pyramin excretion curve with increasing thiamine intakes is difficult to evaluate at this time. Under certain conditions, the pyramin excretion may, temporarily at least, greatly exceed 400 γ. These special conditions will be discussed in subsequent publications.

Excretions of Thiamine and Pyramin As Indicators of Thiamine Intake—It would be highly desirable to evaluate the relative merits of thiamine and pyramin excretions as indices of thiamine intake. At present there are no statistical methods which allow the thiamine data to be used in a strict
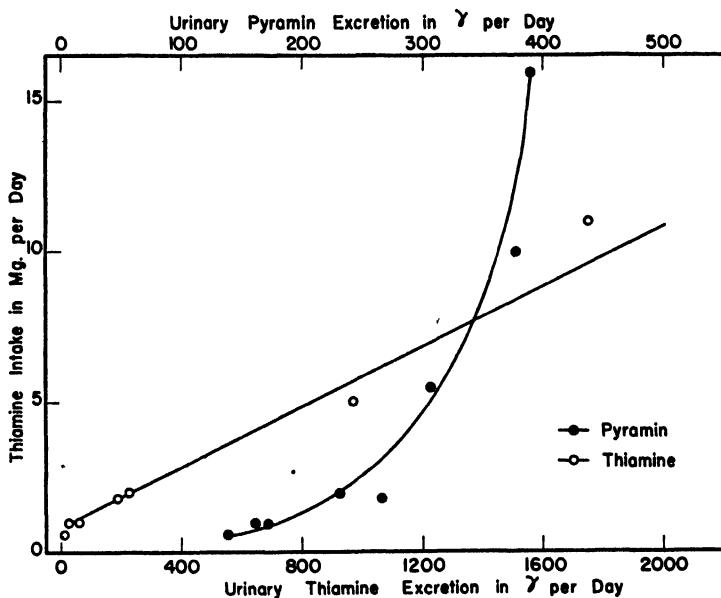
FIG. 2. The excretion of thiamine and pyramin with different thiamine intakes. The data for these curves are from Tables I and II.

analysis of variance. Several attempts at comparison of these two measures, however, have been tried.

In the first of these methods, the thiamine and pyramin excretion values have been plotted against the thiamine intake. At daily thiamine intakes of 2 mg. or less per day two substantially straight lines result, whose slopes on an equimolar basis are not significantly different. The correlation between the corresponding intake and excretion values should indicate how directly the excretion measure is related to the thiamine intake. The measure with the higher correlation coefficient can be taken to be the more directly related to the thiamine intake.

With the mean excretion values of twenty-two individuals who have been maintained on levels of 0.6, 1.0, 1.8, and 2.0 mg. of thiamine per day (Table III), the correlation coefficient ((16) p. 138), r_1, relating thiamine excretion to intake, was +0.88, and the one relating pyramin excretion to thiamine intake, r_2, was +0.93. The pyramin excretion data show the higher correlation coefficient, but with twenty-two cases the difference between the two is non-significant. The difference is still found to be non-significant when all of the original data are used.

Another approach involves the use of Hotelling's t test, which is specifically designed to show which of two variables is best suited for the prediction of a third (1). When the calculations for this test are made, it is again found that pyramin excretion is better, but not significantly so, than thiamine for the prediction of thiamine intake.

Other methods of comparison have also been studied. In one of these, the variations in the excretion values at each intake level have been expressed in terms of thiamine intake by means of the slopes relating the excretion of thiamine and pyramin (expressed in equimolar terms) to thiamine intake. This procedure indicates that the relative superiority of the one excretion test over the other is dependent upon the intake level, but again exact comparisons are difficult.

Below a thiamine intake of about 0.7 mg. per day, the thiamine excretion approaches zero. Statistically these values are very constant, but experimentally not very useful. At these same levels of intake the pyramin excretion values are easily measurable. At thiamine intake levels close to 1.0 mg. of thiamine per day both thiamine and pyramin excretion values are equally reliable as indicators of thiamine intake. At thiamine intakes below 1.0 mg. of thiamine per day the thiamine excretion values are statistically less variable than the pyramin. At thiamine intakes above 1.0 mg. per day the pyramin excretion values are less variable and thus more reliable than the thiamine values.

One of the great drawbacks in the use of thiamine is that the excretion level is highly characteristic of the individual. This property is apparent at the intake levels ordinarily encountered in the normal American dietary, which makes it difficult to compare the data of one group with that from another. Except at intake levels between 0.7 and 1.0 mg. of thiamine per day, the pyramin excretion is slightly more sensitive to intake than is thiamine excretion. Consequently, at intake levels below 1 mg. of thiamine but above 0.7 mg. per day, it is better to use the thiamine excretion as an indicator of the intake. From 1 mg. up to about 5 mg. it is better, however, to use the pyramin excretion. Unless the intake level is known, it is rather difficult to determine which of the two indices is the better. Under such conditions and as a means of improving the accuracy of the intake predic-

tion, it is advisable to determine both excretion compounds.   A multiple regression equation ((16) p. 340) or discriminant function (2) may be set up which in turn can be converted to

$$\text{Mg. thiamine intake} = -0.65 + 0.0086 \text{ pyramin} + 0.0027 \text{ thiamine}$$

TABLE IV

Original Urinary Thiamine and Pyramin Excretion Data of Individual Subjects Maintained on Constant Thiamine Intake for 8 Months

The first six men received 2 mg. of thiamine per day throughout this period, while the other six received 1 mg. B_1 represents the daily thiamine excretions in micrograms and P, the daily pyramin excretion in micrograms of 2-methyl-4-amino-5-ethoxymethylpyrimidine hydrochloride.

Subject		Time of urinary collection												Mean
		December			January	March	April	June	July					
D	B_1	276	294	350	433	398	348	168	381	330	353	276	333	328
	P	230	282	240	228	227	250	142	236	194	211	264	248	229
C	B_1	82	87	88	123	140	164	138	91	128	87	104	86	110
	P	275	290	238	236	216	273	221	244	220	231	270	245	247
B	B_1	231	141	158	238	336	416	500	294	364	296	318	316	301
	P	266	228	195	220	243	245	220	242	185	261	204	235	229
W	B_1	128	191	109	167	180	318	140	112	206	209	153	234	179
	P	238	249	219	203	268	264	207	227	191	211	202	253	228
H	B_1	148	122	138	225	100	176	295	110	193	163	152	198	168
	P	231	306	246	280	272	215	205	219	216	248	247	243	244
Pe	B_1	114	128	101	183	200	191	307	252	193	156	113	134	173
	P	226	278	222	234	296	261	250	272	205	254	209	272	248
A	B_1	117	75	79	105	119	136	119	74	88	94	68	64	95
	P	230	211	190	202	219	194	139	187	130	169	157	174	184
Pa	B_1	36	21	39	46	45	48	62	38	34	32	22	22	37
	P	158	214	162	170	186	185	167	165	112	162	170	142	166
R	B_1	62	52	60	69	97	78	81	55	57	52	40	38	62
	P	165	193	181	157	179	188	142	167	118	159	169	150	164
E	B_1	60	48	53	67	77	81	135	40	38	40	38	42	60
	P	173	220	153	152	192	186	147	146	128	148	178	178	167
M	B_1	85	83	86	122	90	73	70	16	36	38	44	41	65
	P	183	243	210	245	205	121	138	174	104	178	198	179	182
S	B_1	55	46	49	62	76	48	60	45	48	52	40	41	52
	P	173	200	185	188	206	130	143	181	127	188	173	146	170

where thiamine and pyramin are the urinary values for these compounds expressed as indicated above.   The constants in this equation have been calculated from the data in Table IV.   In this equation pyramin is weighted twice as heavily as thiamine, which again indicates that it is a slightly more reliable indicator of thiamine intake (2).   It must be emphasized that all

of the equations given in this paper have been secured from our data and are meant to hold only for our set of conditions, and must be rigidly checked before they are used under any other circumstances.

DISCUSSION

In the past, surprisingly little work has been done with pyramin, especially in prolonged experiments. Pollock, Ellenberg, and Dolger (14) were probably the first to make actual analyses of the pyramin content of the urine (called by them "pyrimidine"). They suggested that the urinary thiamine excretion represented only the immediately preceding vitamin intake, whereas the pyramin excretion more faithfully mirrored the body stores of thiamine. Since the urinary pyramin excretion increases after the injection of 100 mg. of thiamine, they postulated that pyramin is derived from thiamine.

Wertz and Mitchell (17) used the pyramin values expressed as thiamine equivalents, plus the actual thiamine values as a measure of the "total" thiamine excretion. Although their experiments were of only a few days duration, there is an indication that at the lower levels of thiamine intake a greater fraction of the intake can be accounted for on the basis of "total" urinary thiamine excretion than at the higher levels of intake. Our work confirms and supplements this. Fig. 3, which is based on our data, shows that with increasing thiamine intakes the excretion of thiamine increases, but tends to reach a plateau at about 13 per cent of the intake. When the pyramin content of the urine is added to this, the total per cent of the intake accounted for in the urine decreases as the intake increases. Even at the lower dietary intakes, a maximum of 42 per cent of the intake was accounted for as the sum of pyramin and thiamine, whereas at levels of intake of 1 mg. only 35 per cent was accounted for. There is no indication as to the fate of the remaining thiamine. Nothing can be said about the stability of pyramin in the body until it is isolated and characterized. The pyrimidine ring in thiamine may be broken down by the body to compounds other than pyramin.

Gorham, Abels, Robins, and Rhoads (5) found a marked increase in the pyramin excretion following the injection of 5 mg. of thiamine, whereas the pyramin excretion was almost unaffected by the injection of 5 mg. of 2-methyl-4-amino-5-methoxyethylpyrimidine (sic.). Their work also brings out the greater variation in the excretion of thiamine, when compared to that of pyramin.

Regardless of whether thiamine or pyramin is used, a comprehensive statistical evaluation of the urinary excretion is necessary before any assessment can be made of this technique as a measure of vitamin requirement. Many workers have come to conclusions on the human vitamin

requirement based only on unappraised urinary excetion data. Only one such example will be mentioned to illustrate a common fallacy. Mason and Williams (9) increased stepwise the thiamine intake from 0.4 up to 2.0 mg. per day. They noticed a "marked" change in the slope of the urinary thiamine excretion when the intake was increased from 0.8 to 1.0 mg. This led them to conclude "that a level of intake of 800 micrograms was just enough for physiologic purposes and that above this level a greater amount was excreted as surplus" (p. 251). Statistical examination of their data fails to show any significant change in the slope of the urinary excretion
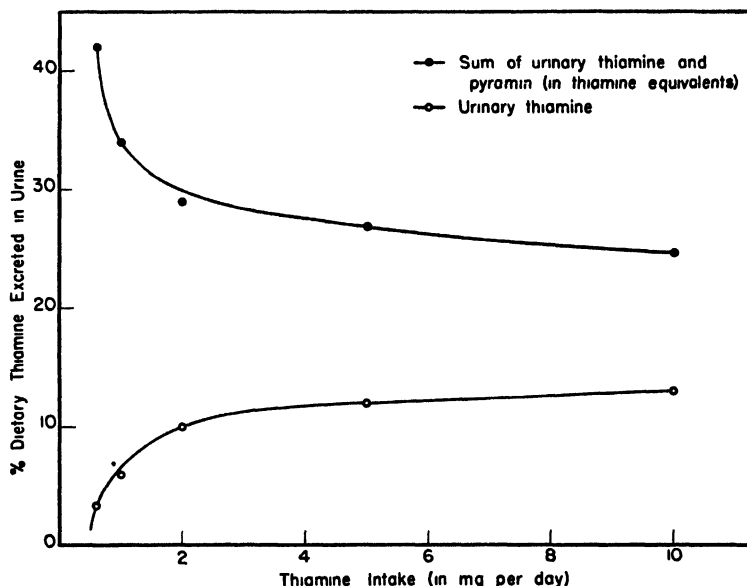
FIG. 3. The recovery of thiamine and pyramin in the urine at different levels of thiamine intake. All values are based on the stabilized excretion values and represent the averages of the four or more men listed in Tables I and II.

curve at this point. In fact, the *total* change in the excretion values at the two intakes is just barely significant ($p = 0.02$), even when advantage is taken of paired variance assumptions.

Various workers have argued strongly for the use of the urinary excretion of thiamine as an index of the nutritional status of the individual. The supporting data consist primarily of a number of isolated short time experiments which were poorly controlled more often than not. In spite of the many analyses of urinary thiamine that have been reported by many workers, practically no one has attempted a statistical analysis to establish the possible significance of the data. The result has been a neglect of some

of the basic facts. Excretion equilibrium at a given thiamine intake level requires many days. We have as yet no proper information as to variations in the rate of attainment of equilibrium. But even when equilibrium has been established at a highly constant thiamine intake, there are, as we have shown, very large consistent individual differences and considerable day to day variations. Marked inter- and intraindividual variations in thiamine excretion associated with a constant thiamine intake have been noted without statistical analysis by Wertz and Mitchell (17). Limitation of analysis to a casual comparison of group averages has obscured the question of the meaning of the individual estimation and has led to unwarranted optimism.

The facts are that one "normal" person may excrete twice or even 3 times as much thiamine as another "normal" person on exactly the same diet. In any one day these differences are apt to be considerably larger than the mean differences. Finally, we may note that when the thiamine intake is changed the rate of response in urinary excretion is such that only half the change is completed in 10 days (Fig. 1). This is true for both thiamine and pyramin excretion. Obviously this fact is of great importance in all experiments relating thiamine excretion to intake and attempts to interpret thiamine "load" or "saturation" tests. Melnick (11) has criticized some of our earlier reports on thiamine excretion associated with low thiamine intakes (7) as being "too low." Our reported excretions, being achieved at the true plateau level, are naturally lower than the values obtained only a few days after the change from a high to a low intake.

In this paper we have discussed only "normal" young men, that is young men with no history, signs, or symptoms of nutritional, digestive, or metabolic peculiarities. We can only believe that the variations in a less rigidly "normal" and homogeneous population must be more than with our subjects.

SUMMARY

1. Thiamine and pyramin excretion values from twenty-two normal young men maintained for prolonged periods on very carefully controlled thiamine intakes of 0.6, 1.0, 1.8, and 2.0 mg. per day have been studied statistically.

2. Thiamine excretion values appear in a general way to be linearly related to the thiamine intake, but are also highly characteristic of the individual. One "normal" individual may excrete twice as much as another "normal" individual on the same thiamine intake. The interindividual, intraindividual, and random variabilities occurring in the thiamine data increase markedly (but not quite in direct proportion) as the excretion values increase. Strict analysis of variance is almost impossible with these data.

3. The relationship between pyramin excretion values and thiamine intake is exponential, with the curve approaching a plateau of about 400 γ of pyramin at very high intake values. In the region of normal intake levels (1 to 2 mg. per day) the relationship is very close to linear. The variabilities are quite homogeneously distributed, a fact which greatly facilitates statistical treatment of the data.

4. Several advantages appear to be secured from the use of pyramin over the thiamine excretion data: (a) greater ease of statistical treatment of the data, with a corresponding increase in certainty of the results; (b) thiamine excretion values are subject to large, consistent, individual differences, while in the pyramin data the individual differences are very small; and (c) at low levels of thiamine intake the excretion of pyramin is still determinable, whereas the excretion of thiamine itself becomes zero.

5. When the thiamine intake is increased from 1 to 2 mg., it requires a period of about 6 weeks for thiamine and pyramin urinary excretion values to come to equilibrium with the new intake level. Half of this change occurs during the first 10 days.

6. The necessity for statistical treatment of excretion data and, further, the necessity for recognizing the nature of the variabilities involved in this data have been pointed out.

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THE CHEMISTRY OF MELANIN

II. THE OXIDATION OF DIHYDROXYPHENYLALANINE BY MAMMALIAN DOPA OXIDASE*

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Raper and Speakman (1, 2) have shown that hallachrome (I) is formed from 3,4-dihydroxyphenylalanine in the presence of potato, fungus, and
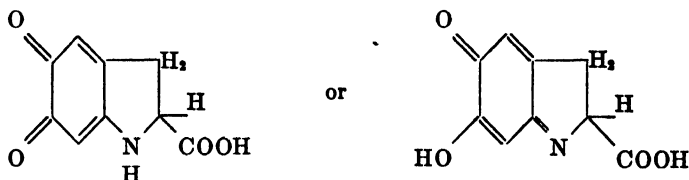
(I)

meal worm tyrosinases. Hogeboom and Adams (3) have isolated a 3,4-dihydroxyphenylalanine oxidase (dopa oxidase) from the Harding-Passey melanoma. In the present study the oxidations of dihydroxyphenylalanine by melanoma dopa oxidase and by mushroom tyrosinase have been compared spectrophotometrically. It has been found that hallachrome is formed in the presence of mammalian dopa oxidase.

EXPERIMENTAL

A mammalian dopa oxidase was prepared by the method of Hogeboom and Adams (3) from the S91-dba mouse melanoma. The tumor material was generously supplied by Dr. Glenn H. Algire of the National Cancer Institute. To measure the activity of the preparation, 0.5 ml. of the enzyme was mixed with 2.5 ml. of phosphate buffer at pH 6.8, containing 0.5 mg. of l-3,4-dihydroxyphenylalanine (Hoffmann-La Roche). 3 atoms of oxygen were consumed per molecule of substrate in 100 minutes. In 600 minutes, 4 atoms of oxygen were consumed. The experiment was terminated at 1000 minutes, when 4.2 atoms of oxygen had been consumed. This determination was carried out at 31.7°; the manometric technique has already been described (4).

Tyrosinase was prepared from *Psalliota campestris*, according to the procedure of Ludwig and Nelson (5). The sample contained 273 cate-

* The first article in this series is in press (*Ann. New York Acad. Sc.* (1947)).

cholase units per ml., or 90 units per mg. of dry weight, as determined by the method of Miller and coworkers (6).

To follow the course of the enzymic reactions by means of the spectrophotometer (Beckman), continuous readings were made and the transformations of the chromophores involved were quantitatively recorded. In applying this technique, 3.0 ml. of buffer solution were measured into a 1.0 cm. quartz spectrophotometer cell (comparison cell), and 3.0 ml. of the same buffer containing dihydroxyphenylalanine were measured into another cell. The absorption spectrum of the substrate was then determined as a check upon the concentration and freshness of the solution. Slit width was maintained at 0.3 mm. and widened at the short wavelengths only as much as required by the decreasing transparency of the comparison solution. The tyrosinase was diluted so that 0.1 ml. contained the desired number of units. Since the dopa oxidase was not of sufficient strength to permit this, the volume of the enzyme used was varied up to 0.3 ml. The enzyme was added to the comparison cell and the mixture carefully stirred. The same volume of enzyme was then added to the cell containing dihydroxyphenylalanine, the time was recorded, and the mixture again carefully stirred. Density values were continuously read, scanning the field from the long to the short wave-lengths at intervals which varied from 2.5 to 20 mμ, depending upon the character of the region. From 3 to 5 minutes were required to scan the range between 400 and 230 mμ. When desirable, the densities at specific wave-lengths were followed continuously. All experiments were conducted at a room temperature of 28–29°.

Results

The spectrophotometric course of the oxidation of varying concentrations of dihydroxyphenylalanine in the presence of varying amounts of tyrosinase was determined as a standard against which the action of dopa oxidase could be compared. In Fig. 1 is depicted the sequence of absorption spectra observed when 3.0 ml. of a substrate solution containing 0.008 mg. of dihydroxyphenylalanine per ml. were oxidized in the presence of 0.27 (A), 1.4 (B), and 9.0 (C) catecholase units of mushroom tyrosinase. The absorption spectrum of dihydroxyphenylalanine, maximum = 280 mμ, log e = 3.43, was replaced by a new and more intense maximum at 305 mμ when 9.0 units of enzyme were employed. In the presence of 0.27 and 1.4 units, an incomplete progression toward this curve could be observed.

The results obtained when 3.0 ml. of substrate solution containing 0.024 mg. of dihydroxyphenylalanine per ml. were oxidized in the presence of 1.4 (A), 9.0 (B), and 13.5 (C) catecholase units are presented in Fig. 2.

The absorption spectrum of dihydroxyphenylalanine was again found to be replaced by a new spectrum with a maximum at 305 mμ. The height and speed with which the new maximum developed were a function of the enzyme concentration. In the presence of 13.5 units of enzyme, the chromophore reached its greatest concentration in 15 minutes. At this point the solution was bright red; the color thereafter changed to purple and finally to brown, corresponding to the formation of synthetic
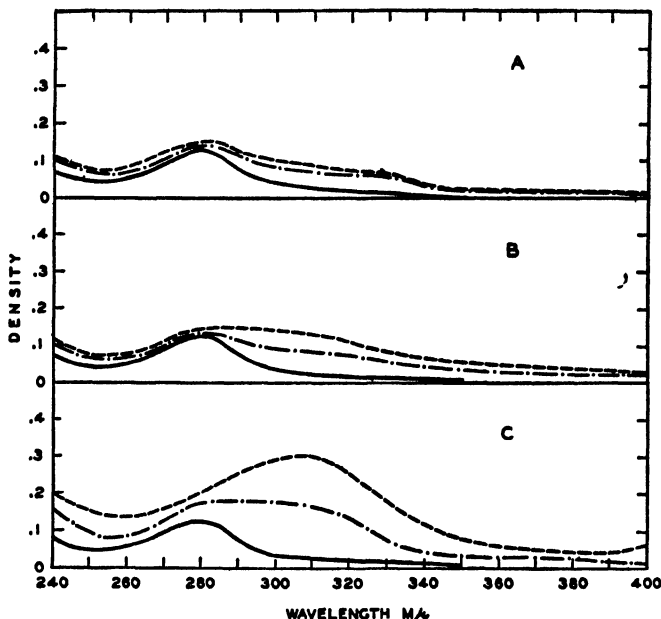
FIG. 1. Spectrophotometric course of the oxidation of 3,4-dihydroxyphenylalanine in the presence of tyrosinase. A, 0.008 mg. per ml and 0.27 catecholase units at (reading up) 0, 10, and 35 minutes; B, with 1.4 catecholase units at 0, 10, and 50 minutes; C, with 9 0 catecholase units at 0, 2, and 11 minutes.

dopa melanin. During these changes the absorption spectra gradually approached curves indicating general absorption.

The dopa oxidase preparation proved to be much less active than the partially purified tyrosinase. When 3.0 ml. of a substrate solution containing 0.024 mg. of dihydroxyphenylalanine per ml. were oxidized in the presence of 0.1 ml. of dopa oxidase (Fig. 3, B), an incomplete sequence was obtained, roughly approximating the oxidation of a like amount of dihydroxyphenylalanine in the presence of 1.4 units of tyrosinase. By increasing the amount of enzyme employed to 0.2 ml., the sequence of reactions progressed to a clear development of an absorption spectrum

with a maximum at 310 mμ'(Fig. 3, C). The optical properties of the reaction mixture were such that no measurements could be obtained below 285 mμ.   A similar development of an absorption spectrum with a maximum
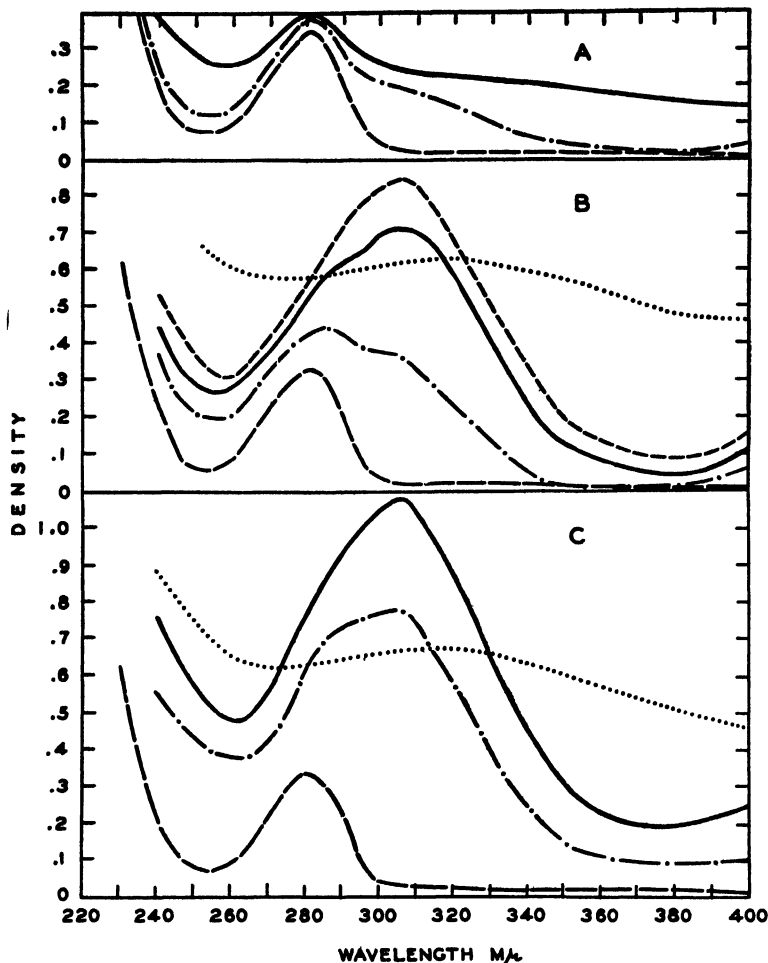
FIG. 2. Spectrophotometric course of the oxidation of 3,4-dihydroxyphenylalanine in the presence of tyrosinase.   A, 0.024 mg. per ml. and 1.4 catecholase units at 0 and 28 minutes and 19 hours; B, with 9.0 catecholase units at 0, 2, 5, and 21 minutes and 18 hours; C, with 13.5 catecholase units at 0, 1, and 15 minutes and 18 hours.

at 305 to 310 mμ was observed when the concentration of the substrate was 0.008 mg. per ml.   By advancing the ratio of enzyme to substrate concentration to 0.3 ml. of enzyme per 3.0 ml. of solution containing 0.008 mg. of dihydroxyphenylalanine per ml., a new effect was observed.   An

intermediate absorption spectrum with a maximum below 285 mμ quickly developed. This gradually disappeared and was replaced by the previously observed maximum at 305 to 310 mμ (Fig. 3; A).
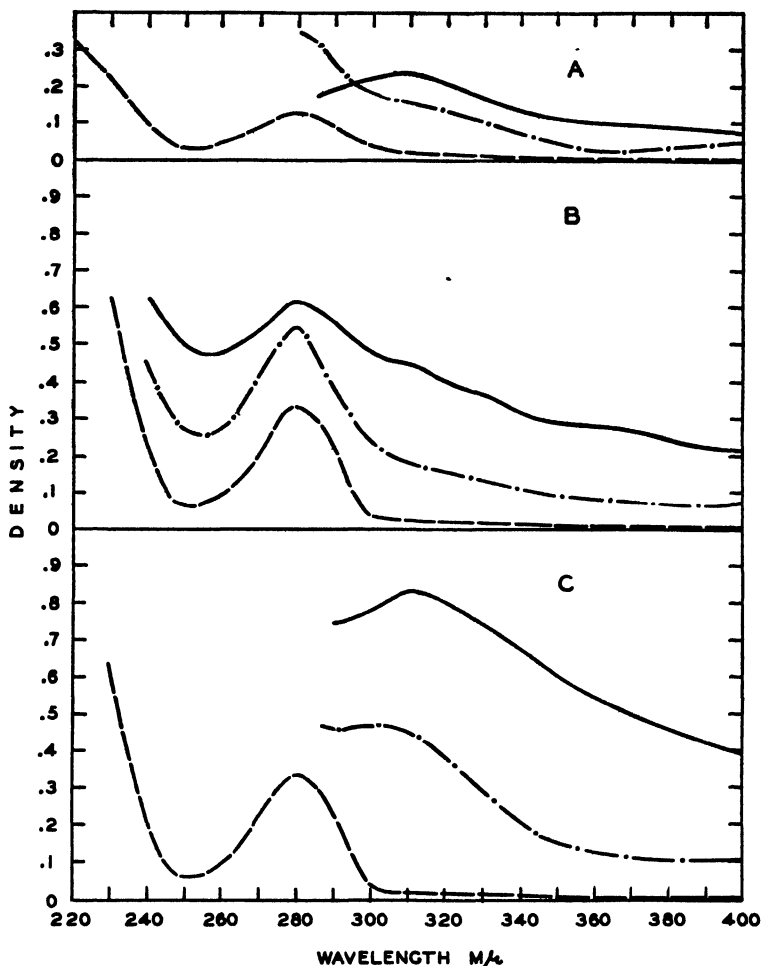
FIG. 3. Spectrophotometric course of the oxidation of 3,4-dihydroxyphenylalanine in the presence of dopa oxidase. A, 0.008 mg. per ml. and 0.3 ml. of enzyme at (reading up) 0, 15, and 120 minutes; B, 0.024 mg. per ml. with 0.10 ml. at 0, 8, and 150 minutes; C, 0.024 mg. per ml. with 0.2 ml. of enzyme at 0, 15, and 150 minutes.

DISCUSSION

Dihydroxyphenylalanine is known to be oxidized to a brown pigment in the presence of both mammalian (3) and non-mammalian (1) phenol-

oxidases. When investigated, these processes have been shown to require approximately 4 atoms of oxygen per molecule of substrate (3, 7). The initial sequence of reactions taking place when dihydroxyphenylalanine is oxidized in the presence of tyrosinases has been worked out by Raper (2). It has not been proved that a similar sequence occurs when synthetic dopa melanin is formed by mammalian dopa oxidase. In the present investigation, red, and finally brown, pigment was observed to form when dihydroxyphenylalanine was oxidized in the presence of either mushroom tyrosinase or a mammalian dopa oxidase. Since the red pigment is characterized in both instances by maximum absorption at 305 to 310 mμ, it is probable that hallachrome is formed from dihydroxyphenylalanine by both enzymes.

The enzyme preparations were of different relative purities. No conclusions regarding the relative rates at which the red pigment formed may be drawn. When the ratio between the concentrations of dopa oxidase and the substrate was high, a chromophoric substance intermediate between dihydroxyphenylalanine and hallachrome accumulated. While several explanations for this change in the relative rates of the consecutive reactions involved are evident, no decision is possible at present.

The slight shift in the position of the absorption maximum of the red pigment between 305 and 310 mμ (cf. Figs. 2, C and 3, C) may be ascribed to the metachromatic effect (8) arising from differences in quantity and kind of protein in the two enzyme preparations.

SUMMARY

1. An absorption maximum at 305 to 310 mμ, characterizing hallachrome, developed when dihydroxyphenylalanine was oxidized in the presence of either mushroom tyrosinase or melanoma dopa oxidase.

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A COMPARATIVE STUDY OF THE METABOLISM OF α-ALANINE, β-ALANINE, SERINE, AND ISOSERINE

I. ABSORPTION FROM THE GASTROINTESTINAL TRACT

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One of the earliest suggestions that the rate of absorption from the intestine might not be the same for all amino acids came from indirect evidence. The amino acid nitrogen of the blood of dogs and rabbits showed a more rapid increase after the oral administration of glycine and alanine than after similar administration of glutamic and aspartic acids (1). It was suggested that the naturally occurring dicarboxylic amino acids might be less rapidly absorbed. Studies of the changes of the non-protein nitrogen of the blood after amino acids were fed to rabbits led Johnston and Lewis (2) to suggest that the rate of absorption of amino acids from the intestine might be one of the important factors in the rate of metabolism of the amino acids.

The Cori technique (3) permits an accurate determination of the material absorbed from the gastrointestinal canal of rats and the use of small animals of this type has made possible such a study with the use of relatively small amounts of amino acids. Wilson and Lewis (4) extended the preliminary experiments of Cori (5), who studied glycine and alanine, to include alanine and a number of other amino acids. The absorption of most of the other amino acids of the protein molecule has now been investigated by this procedure.

The purpose of the present study was to determine the effect of stereochemical differences on the rate of absorption of amino acids, as exemplified by an investigation of the comparative behavior of *l*-, *d*-, and *dl*-alanine. The influence of a shift in the position of the amino group has also been determined by a comparison of *dl*-alanine with the naturally occurring β-alanine and of *dl*-serine with *dl*-isoserine. The comparison of serine with alanine and of isoserine with β-alanine should afford information on the effect of the introduction of a hydroxyl group into the molecule of the amino derivatives of propionic acid.

The general procedure was that of Cori (3) as modified by Wilson and Lewis (4) for the study of the absorption of amino acids. Male white rats, whose weight after a 24 hour fast was 115 to 135 gm., were used. In order to insure uniformly good nutritive condition, they were all fed a commercial rat diet (Rockland or Purina chow) for 7 to 21 days prior to the fasting period. The preliminary period of fasting (24 hours), the methods of administration of the amino acids, and the preparation of the solutions for analyses of the gastrointestinal contents were similar to those used previously (4).

The period allowed for absorption was in all cases the same, 3 hours. It should be noted also that, in all experiments, the amounts of amino acids fed were in considerable excess of the amount which could be absorbed over the total 3 hour period. The amino acids were in aqueous solution as such rather than as the sodium salts fed in certain of our earlier experiments (4). It was possible to improve our procedure so that the period during which the gastrointestinal tract remained frozen in the ice box after removal from the body and prior to the analyses was reduced to 2.5 hours.

The amino acids were obtained from commercial sources except β-alanine and isoserine which were synthesized in this laboratory. In the analysis of β-alanine by the Van Slyke volumetric amino nitrogen procedure, a reaction time of 15 minutes was required instead of the usual 3 for complete reaction. This period was used in the analyses of gastrointestinal contents in those experiments in which β-alanine was fed. Isoserine gave somewhat more than the theoretical amino nitrogen in 3 minutes and it was necessary to use the proper correction factor for this amino acid. All compounds were analyzed for total and amino nitrogen with satisfactory results.

The d- and l-alanine were obtained through the cooperation of Dr. Max S. Dunn of the University of California at Los Angeles, in whose laboratory their specific rotations, $-13.57°$ and $+13.83°$ respectively, were determined under standard conditions ($t = 25°$; $\lambda = 5893$ A; solvent, 5.97 N hydrochloric acid; concentration of amino acid, approximately 2 gm. in 100 ml. of solvent). Other samples of the optical isomers of alanine, secured through Hoffmann-La Roche, Inc., showed satisfactory purity on analysis, but their optical activity was not examined. The samples from both sources behaved similarly in the biological studies.

To test the accuracy of the procedure, a solution of dl-alanine of approximately the same concentration as that used in the absorption studies was fed to three rats and the animals were killed as rapidly as possible. Not more than 4 minutes elapsed between the introduction of the solution into

the stomach and the placing of the excised gastrointestinal tract in the freezing chamber. Recoveries of alanine (as amino nitrogen) were 93, 94, and 93 per cent of the amount fed. These recoveries are similar to those of Butts, Dunn, and Hallman (6) who obtained 91.5 per cent in similar control experiments.

Since the amount of amino acid absorbed is determined as the difference between the amount of amino nitrogen fed and that remaining in the tract at the end of the absorption period, it is necessary to correct the latter value for the residual amino nitrogen of the tract of the fasting rat as described previously (4). This correction was determined with two series of animals, a summer series and a fall and winter series. In the summer group, twelve animals gave an average of 6.9 mg. of residual amino acid nitrogen per 100 gm. of body weight, with a range of 5.0 to 9.7 (only two values exceeded 7.9), and in the fall-winter group of twenty animals, slightly lower values were obtained, 4.4 mg. with a range of 2.4 to 7.1 mg. (only one value above 6.5). These values were used as correction factors in the corresponding series of experimental animals. These control values are slightly higher than those obtained previously in this laboratory (4), but in those series, also, somewhat higher residual amino nitrogen values were observed in the summer series.

The results obtained are presented in condensed form in Table I. With dl-alanine, a summer series of sixteen animals and a second (fall-winter) series of fourteen animals were studied. Absorption coefficients of 79 (range, 64 to 96) and 82 (range, 77 to 92) mg. per 100 gm. of rat per hour were obtained. All subsequent experiments with other amino acids were in the fall-winter series. The absorption coefficient of dl-alanine was quite high and in general, similar to but slightly higher than the value (72) obtained previously in this laboratory.

The rate of absorption of the l isomer of alanine was determined in seventeen animals, twelve of which received the amino acid obtained from Hoffmann-La Roche, Inc., and five that from Dr. Dunn. The coefficients of absorption of the two groups were 83 and 79 respectively, with a mean for all seventeen animals of 81.5. In the studies with d-alanine, the animals receiving the Hoffmann-La Roche and the Dunn preparation numbered thirteen and five respectively. The coefficients of the two groups, 62 and 59, were not significantly different. The mean of all eighteen animals was 61.6. d-Alanine appears to be absorbed at a slightly lower rate than the l isomer or racemic alanine.

β-Alanine, which because of its slightly greater solubility could be fed at slightly higher levels than dl-alanine, was absorbed at a definitely lower rate (51.4). Somewhat greater individual variations were noted in the experiments with β-alanine than were observed in the dl- and l-alanine

series. A similar variation was observed in the *d*-alanine series. It seems probable that, when the rate of absorption is lower, individual differences may be more evident.

The rate of absorption of serine (67.1), while definitely lower than that of either *dl*- or *l*-alanine, was considerably greater than that of *β*-alanine.

TABLE I

Statistical Comparison of Rates of Absorption of Amino Acids from Gastrointestinal Tract during 3 Hour Periods of Rats Fasted for 24 Hours

In Column A, the rate of absorption of the amino acids under consideration is compared statistically with that of *dl*-alanine; in Column B, with that of *d*-alanine; in Column C, with that of *l*-alanine; and in Column D, with that of *dl*-serine.

No. of rats	Amino acid	Absorption coefficient, per 100 gm. per hr.			S^{2*}	t† for difference of means			
		Maximum	Minimum	Mean ($\bar{X}$)		(A)	(B)	(C)	(D)
		mg.	*mg*	*mg.*					
30	*dl*-Alanine	96	64	80 5+	52.3				
18	*d*-Alanine	78	40	61.6	74.5	8.2		**8.0**	
17	*l*-Alanine	92	73	81.5+	21.9	**0.50** ($P > 0$ 6)			
13	*β*-Alanine	60	34	51 4	61.1	11.8	3.4 (0.001 > P > 0.01)	**13.2**	
12	*dl*-Serine	75	54	67.1	37.5	5.7			
12	*dl*-Isoserine	19	10	14.1	8.4	**36.3**			**32.0**

* S^2 is the unbiased estimate of the population variance $\left(S^2 = \dfrac{\Sigma(X - \bar{X})^2}{N - 1} \right)$.

† Values of t not in bold-face are t values as calculated by "Student"-Fisher. In the case of the values in bold-faced type, the ratio of estimated variances was too great (for the Snedecor F (variance ratio), P <0.10) to assume that both samples came from equally variable populations and hence the usual "Student"-Fisher test for the comparison of means is not applicable. The samples here are smaller than one ought to have for the use of the large sample test in which the variance of the difference of sample means is simply estimated as the sum of their estimated variances There is, however, an exact test (Scheffé) available for such cases, and this gives a t which obeys the "Student"-Fisher law but with degrees of freedom 1 less than the smaller sample size (7). The bold-face values of t are of this latter kind. Values of P are given whenever they are >0.001.

The coefficient of isoserine (14.1), on the other hand, was much lower than that of any other amino acid studied in these experiments. This is to be contrasted with the very rapid absorption of alanine and the good absorption of serine. Even the more slowly absorbed *β*-alanine (in which the amino group is in the same position as in isoserine) was absorbed at a rate approximately 4 times as great as that of isoserine.

Because of the limited solubility of isoserine in water, the amounts which could be dissolved in the 2 ml. of solution administered were small (49 to 68 mg. per 100 gm. of rat), amounts definitely lower than those of the other amino acids studied (226 to 419 mg.). Although amino nitrogen was still present in the contents of the gastrointestinal canal in considerable amounts at the end of the 3 hour absorption periods, it seemed possible that the low rate of absorption might be related, in part, at least, to the small quantity of the amino acid presented for absorption in the tract. As a control, a series of animals received dl-alanine in amounts similar to the amounts of isoserine fed. At the end of a 3 hour absorption period, the alanine had been completely absorbed, a finding which was to have been anticipated if the absorption had proceeded at a rate comparable to that of the other experiments with alanine. The poor absorption of isoserine can hardly be attributed to the small amounts of the amino acid which could be fed.

The data obtained were analyzed by the Statistical Research Laboratory of the University of Michigan. We wish to express our appreciation to Professor Cecil C. Craig, Director of the Laboratory, for his aid in the interpretation of the data. As shown in Table I (Columns A, B, C, and D), all of the differences are of statistical significance except the differences between the absorption coefficients of dl- and l-alanine.

The observation that dl- and l-alanine were absorbed at essentially the same rate is in agreement with previous work[1] from this laboratory (4). A study of the literature fails to reveal any real differences in the rate at which the "natural" optically active isomers and the racemic amino acids are absorbed. In previous studies from this laboratory (8), the absorption coefficients of the racemic forms of leucine, isoleucine, and valine were found to be the same as those of the l forms. While differences in the coefficients of absorption of racemic and l-tryptophane have been reported as indicating a slightly more rapid absorption of the l isomer (9), the differences are so slight as to make this conclusion a debatable one.

In the present experiments, d-alanine was absorbed at a rate significantly lower than that of l-alanine. We are not familiar with any previous experiments with rats by the Cori procedure in which the rates of absorption of the d and l isomers of pure amino acids are compared.

It was surprising to observe that the presence of d-alanine in the racemic alanine did not lower the rate of absorption of this material as compared with l-alanine. If racemic alanine were an equimolecular mixture of the d and l isomers, its absorption coefficient might be expected to be less

[1] It should be noted that in the earlier papers cited from this laboratory (2, 4, 8), the older amino acid nomenclature is used. d-Alanine of previous experiments (4) is l-alanine according to the nomenclature used in the present paper.

than that of *l*-alanine, since in general the rate of absorption of a mixture is less than the rates of absorption of its components when fed alone (5). If racemic alanine is a compound, rather than a mixture as has been suggested for some amino acids, notably cystine (10), its absorption coefficient might be characteristic and the same as or different from those of the component optically active isomers. It would be of interest to feed amounts of a racemic amino acid such that a considerable amount of unabsorbed acid would remain in the gut after a period of absorption and to determine whether the residual material is racemic (*i.e.*, both isomers have been absorbed to the same extent) or shows some optical activity (*i.e.*, one isomer has been absorbed more rapidly than the other).

The data warrant a brief discussion of the influence of chemical structure on the rate of absorption, as far as concerns the amino derivatives of propionic acid. When the amino group is more remote from the carboxyl group as in β-alanine or isoserine, the absorption coefficient is decreased in comparison with that of the corresponding α-amino acid, alanine or serine. When a second substituent group, in this case a hydroxyl group, is also present in the molecule, the rate of absorption is also decreased, as is evident by a comparison of the absorption coefficients of alanine and serine and of β-alanine and isoserine. The difference between the last pair of amino acids is the most marked of our series.

The values obtained in this series cannot be compared directly with the absorption coefficients obtained by other workers. The conditions of the experiments are so variable (age, weight, sex, period of absorption, etc.) as to make any comparisons of little value. Moreover, as shown previously (4, 8), the rate of absorption is different when the same amino acid is fed either as the free amino acid or as the sodium or potassium salt. The different solubility of the amino acids may also be important. In the present series, the solubility of the acids fed can hardly be a significant factor, except possibly in the case of isoserine, the most poorly absorbed of those studied here.

<div align="center">SUMMARY</div>

1. The rates of absorption of certain amino derivatives of propionic acid from the gastrointestinal tract of young white rats by the technique of Cori have been determined. The acids studied may be arranged in the following descending order of absorption coefficients: *l*-alanine and *dl*-alanine (essentially the same), *dl*-serine, *d*-alanine, β-alanine, and *dl*-isoserine. Statistical analysis has shown that, except in the case of *dl*- and *l*-alanine, the differences are significant.

2. A comparison of the rates of absorption of α-alanine and β-alanine and of serine and isoserine indicates that, as the amino group was removed

from the carboxyl, the rate of absorption was decreased. Similarly the replacement of a hydrogen by a hydroxyl group, as shown by a comparison of alanine and serine and of β-alanine and isoserine, resulted in a decreased rate of absorption.

3. d-Alanine was somewhat less readily absorbed from the gut than was its stereoisomer, l-alanine.

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A MICROMETHOD OF PHOSPHATE DETERMINATION

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The proposed method is at least twice as sensitive as Bodansky's (1) stannous chloride method and about 15 times more sensitive than the Fiske-Subbarow (2) method. A suitable concentration range for routine work is 0.02 to 0.2 mg. per liter of phosphorus in the sample-reagent mixture

The method is based on the color change of a dye (quinaldine red) solution, presumably due to the formation of an insoluble dye-phosphomolybdate complex which remains in suspension.

The determinations can be made with about 1 per cent precision. The drift (change in color intensity with time) is much smaller than in the Fiske-Subbarow method, while the temperature coefficient is slightly larger. The reagents keep well, and the readings of phosphate standards change little from day to day.

On the other hand, the pH adjustments must be more accurate than in the Fiske-Subbarow method.

Good agreement with the amidol-molybdenum blue method has been obtained in the analysis of calcified tissues and serum filtrates (see Table I).

Reagents and Chemical Procedure

Glassware and Its Use—The glassware, except for the colorimeter tubes, was of Pyrex. The water was purified by single distillation through a tin condenser and stored in glass-stoppered Pyrex vessels.

Quinaldine red solutions formed a coating in the burettes which often interfered with proper drainage. The coating was readily removed with detergents, for instance with 50 per cent solution of Aerosol IB (obtained from the American Cyanamid Company, New York).

The reaction vessels and colorimeter tubes were rinsed with concentrated H_2SO_4 after use. Photometer cells with fused on windows were rinsed *quickly* with 50 to 75 per cent H_2SO_4, immediately after with water, and left filled with water for half an hour in order to soak the acid out of the joints. The use of cemented cells is not recommended.

Preparation of Samples for Analysis—The calcified tissues were dissolved in 1 N HCl (0.8 ml. was taken for 3 mg. of enamel or ashed tissue and for 4 mg. of dentin or bone). To each 3 mg. of the dissolved enamel or ash

and 4 mg. of the dentin or bone were added 6.75 ml. of 10 N H$_2$SO$_4$, plus water to make 1 liter. Any undissolved residue was allowed to settle, and the samples (containing 0.4 to 0.5 mg. per liter of phosphorus) were withdrawn from the top.

The blood sera were deproteinized with 9 volumes of 0.55 N CCl$_3$COOH, and the filtrates were diluted ten times with water. The 0.55 N (approximately 10 per cent) acid was prepared from the 20 per cent solution by titrating the latter and diluting accordingly.

Phosphate Standards for Calcified Tissue Analysis—Stock phosphate (100 mg. of P per liter) contained 0.4390 gm. of KH$_2$PO$_4$ and 10 ml. of 10

TABLE I

Quinaldine Red Method Checked against Molybdenum Blue Method

The difference shown is between the quinaldine red and molybdenum blue values, expressed in per cent of the molybdenum blue value.

Description of sample	Phosphorus content		Difference
	Quinaldine red method	Molybdenum blue method	
	per cent	*per cent*	
Mixed dental tissue, ashed before dissolving	14.07	13.94	+0.9
Alkaline ash of dentin-cementum mixture	16.15	15.91	+1 4
Dental enamel, defatted, dried	17.45	17.37	+0.5
Dentin, dried	12.77	12.57	+1 6
Bone, fresh (outer part of calf fibula)	10.66	10.81	−1.4
" " " " " " "	10.45	10.45	0
Human serum	0 00382	0.00381	+0.3
" "	0.00320	0.00319	+0.3
" "	0.00400	0.00396	+0.9
Horse "	0.00339	0 00337	+0.8
" "	0.00300	0.00304	−1.3$_*$
Rabbit "	0 00446	0.00444	+0 4

N H$_2$SO$_4$ per liter. Standards containing 0.3 and 0.5 mg. of P per liter were prepared from stock plus 7 ml. of 10 N H$_2$SO$_4$ per liter.

Phosphate Standards for Serum—For standards containing 0.2 and 0.4 mg. of P per liter, the requisite amount of stock phosphate (*cf.* above) plus 90 ml. of 0.55 N CCl$_3$COOH plus 0.5 gm. of anhydrous Na$_2$CO$_3$ was made up to 1 liter.

Quinaldine Red-Gum Arabic Solution—To 50 mg. of quinaldine red and 25 mg. of gum arabic were added 500 ml. of water, and the flask placed on the steam bath for 1 hour, with stirring at 10 minute intervals. (The dye should dissolve within 40 minutes.) After being cooled immediately under the faucet, the solution was decanted into a glass-stoppered Pyrex bottle. The bottle was kept out of direct sunlight.

Quinaldine red (2-*p*-dimethylaminostyrylquinoline ethiodide) supplied by the Eastman Kodak Company was satisfactory. Several preparations, according to Koenig and Treichel (3), gave slightly higher melting points but no marked difference when used in the reagent solution.

Gum arabic was of the best commercial grade ("U. S. P. select, lumps;" J. T. Baker's pea size; and Penick's "white tears" proved to be satisfactory). Clear and nearly colorless lumps, free from enclosures, were selected and powdered. A "U. S. P. powder" preparation was tried, but double the normal blank reading was obtained.

The reagent is usable for at least 3 to 5 weeks, depending upon the concentration range in which the measurements are made (see Table II).

TABLE II

Extinction Coefficients for 0 to 0.2 Mg. of Phosphorus per Liter

k is the extinction coefficient (average of three replicates). Δ is the percentage deviation from proportionality between *k* and the phosphorus concentration (0.1 mg. per liter taken as the reference).

Description of dye solution	Values of	For phosphorus concentration in mg. per liter				
		0	0.02	0.04	0.10	0.20
Solution I, 1 day old, 23°	*k*	0.0072	0.0440	0.0870	0.2136	0.4470
	Δ		+3.0	+1.8	0	+4.6
" " 17 days old, 23°	*k*	0.0079	0.0413	0.0797	0.2015	0.4168
	Δ		+2.6	−1.1	0	+3.4
" II, 1 day old, 24°	*k*	0.0073	0.0440	0.0821	0.2094	0.4258
	Δ		+5.0	−2.0	0	+1.7
" " 7 days old, 24°	*k*	0.0084	0.0419	0.0814	0.2085	0.4264
	Δ		+0.1	−2.3	0	+2.2
" III, 39 days old, 23°	*k*	0.0122	0.0387	0.0769	0.2170	0.4377
	Δ		−10.8	−11.4	0	+0.8

Perhaps the best period is 1 to 3 weeks after the preparation, when the daily change is slight.

Molybdate-Sulfate—To 250 ml. of 10 N H_2SO_4 were added 8.85 gm. of ammonium molybdate (81.4 per cent of MoO_3) and the solution was made up to 1 liter.

No effects of aging were noticed, except on the temperature coefficient of color development.

Procedure

2 volumes of the sample (or standard) and 2 volumes of the dye solution were measured into a dry or well drained vessel and mixed. 1 volume of the molybdate-sulfate was added next from a transfer pipette and with

continuous stirring, the solution being discharged against the wall of the vessel. The mixture was allowed to stand for 10 minutes before reading.
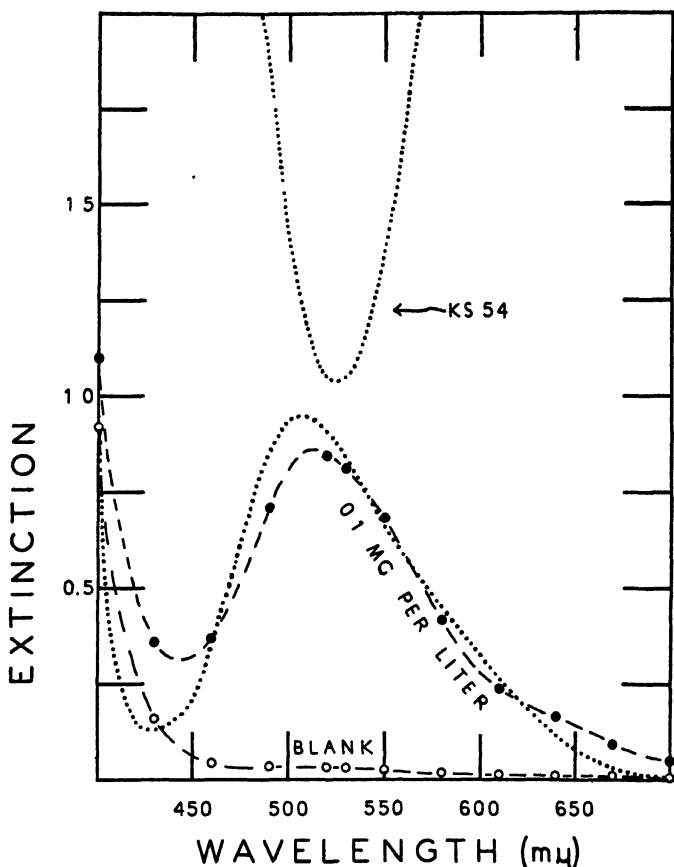
Fig. 1. Extinction curves of the solutions and the light filter. The plotted extinction values of the solutions are extinction coefficients multiplied by 4 (*i.e.* extinctions of 4 cm. deep solution layer). The dashed curves are those of a reagent blank and a 0 1 mg. per liter sample, read in a Coleman spectrophotometer model 11 (band width 35 mμ). The dotted curves represent extinctions of a 0 1 mg. per liter sample and of the filter (No. KS 54) used in the colorimetric readings, calculated from transmittance curves traced in a Hardy spectrophotometer (band width 10 mμ) at the Electrical Testing Laboratories, New York. According to the manufacturer (Klett Manufacturing Company, New York), the transmission maximum of a No. KS 54 filter is near 540 mμ.

The volume of the dye solution was read to 1 per cent (*cf.* p. 452).

The rate of the molybdate addition and mixing influenced the color development. The outflow time of the transfer pipette should therefore be

at least 40 seconds, and the unknowns and standards should be prepared in similar vessels (cf. p. 452).

The time of outflow of Pyrex pipettes usually had to be increased, in order to meet the above requirements, by bringing the pipette tip to a red glow in the gas flame and, if shrunk too far, grinding the end on a carborundum stone.

Measurement of Color Intensity

Absorption curves of the process samples and of the filter used in the colorimetric readings are given in Fig. 1.

Most of the colorimetric readings reported in this paper were made in a Lumetron colorimeter No. 402-E (Photovolt Corporation, New York).

TABLE III

Estimates of Precision

P = the phosphorus concentration in the color mixture; v = the volume of the mixture, t = the time of outflow of the molybdate, n = the number of the replicates; V = the coefficient of variation.

Procedure	P	v	t	n	V
	mg per l.	ml	sec		per cent
Samples mixed in beakers; 3 readings each, in 2 cm. deep Pyrex cell	0 08	25	60	12	0 25
As above, with 2 matched cells; 2 readings each	0 08	25	60	12	0 45
Samples mixed and read in 14.5 mm. tube; 2 readings each	0 2	5	37	10	0 50
As above, but 3 readings each	0 04	5	57	10	0.95

5 ml. volumes were mixed and read in 14.5 mm. tubes; satisfactory precision was obtained down to 0.04 mg. of P per liter (see Table III). More dilute samples and reagent blanks were read in 2 to 5 cm. deep Pyrex cells.

With the Klett-Summerson colorimeter, the precision of the readings was limited by the null-point sensitivity. The sensitivity was increased by using filters of greater over-all transmission, for instance Wratten No. 61 (4). Slightly lower readings of the blank and phosphate samples were obtained with the latter than with the filter shown in Fig. 1.

Estimates of Precision and Sources of Error

Drift and Temperature Coefficient—The extinction readings obtained 10 minutes after mixing, as well as the subsequent drift (increase with time), varied measurably with the temperature. Estimates of these effects, obtained with samples of 0.1 mg. of P per liter and averaged for three temperature intervals, follow.

		20-25	25-30	30-35
Temperature, °C..........	.			
Drift between 10 and 20 min. after mixing, %.		0 65	0.5	0.35
Temperature coefficient of extinction (10 min. reading), % per degree		0.7	0.6	0.4

Smaller drift than the above (0.4 per cent at 25°) and slightly lower temperature coefficient were observed with 5 week-old quinaldine red solutions.

Double the above temperature coefficient, which approached the normal in a week, was observed when freshly prepared molybdate-sulfate was used.

Dye Solution Varied in Amount—As noted before, the quinaldine red solution did not drain very well. The observed error in volume was not more than 1 per cent of the amount measured. In order to estimate its importance, the volume of the dye solution was varied (1.9 and 2.1 instead of 2.0 ml.) and the resulting change in extinction determined.

Per cent change in amount of dye	Per cent change in extinction	
	P = 0.04 mg. per liter	P = 0.2 mg. per liter
−5	+0.7	+0.7
+5	+0.2	+0.5

It appears from these results that the probable effect of the drainage error on the extinction is of the order of 0.1 per cent.

Molybdate Solution Varied in Amount—A series of color mixtures was prepared in which the volume of the molybdate-sulfate was increased by 2.6 per cent (from 1.007 to 1.033 ml.). The resulting average decrease in extinction was 1.6 per cent for the mixtures containing 0.04 mg. of P per liter and 1.3 per cent for the mixtures containing 0.2 mg. of P per liter.

The use of a transfer pipette is specified in the procedure and the observed variation in the volume delivered from a 1 ml. pipette was not over 0.3 per cent. From the above results, the effect of such variation on the extinction is estimated at 0.1 to 0.2 per cent.

Rate of Molybdate Addition—The extinction readings increased with the time of outflow of the molybdate-sulfate (Table IV); the increase was small after the latter reached 1 minute. The following results were obtained with 1 ml. pipettes of varying time of outflow.

It appears from the above that changing the time of outflow from 50 to 51 seconds would increase the extinction reading by about 0.1 per cent.

Estimates of Precision—The colorimetric reading was subject to instrumental and other errors whose estimated total was between 0.5 and 1 per cent, depending upon the color intensity and on the absorption vessel. In general, these errors appeared to be at least as large as the chemical

errors. Four of the experimental estimates of precision are given in Table III. In making the first one, the absorption cell and details of the procedure were chosen with a view to maximum accuracy of the extinction measurements. The third and fourth estimates are examples of the reproducibility possible in a series of rapid analyses performed in colorimeter tubes.

Phosphate Concentration Limits; Aging of Dye Solution—More than a dozen quinaldine red solutions were prepared and tested, mostly in the range of 0.1 to 0.2 mg. of phosphorus per liter. The extinction coefficients obtained with three of the solutions (which contained gum arabic from the same source) over a 0 to 0.2 mg. per liter range are shown in Table II. The values obtained with the other solutions are in general agreement with those in Table II.

Calibration curves plotted from the above data depart measurably from

TABLE IV

Effect of Rate of Molybdate Addition

Outflow time, increased	Per cent increase in extinction	
	P = 0 04 mg. per liter	P = 0.2 mg. per liter
sec.		
From 15 to 22	6.4	6.1
" 22 " 37	9.5	7.7
" 37 " 57	0.8	2.2
" 57 " 150	3 4	4 3

the straight line. The deviations are increased by subtracting the reagent blanks from the k values in Table II.

The extinction coefficients declined, while the reagent blanks showed a definite increase, on aging of the dye solution. The daily change in the age interval of 1 to 3 weeks was too small to affect the reproducibility of readings extending over several hours. After 3 weeks, the downward trend in extinction readings of phosphorus concentrations below 0.1 mg. per liter became more rapid, and the values of Δ (*cf.* Table II) generally too large for accurate work.

The aging of the dye solution was thought to result from gradual destruction of the gum arabic. Other protectors were tried, and a more stable solution was obtained with Tween 20 (p. 457), which after 3 months gave approximately the same calibration curve as a week-old quinaldine red-gum arabic solution.

The limitations of apparatus as well as chemical sources of error (deviations from proportionality, reagent blank) fix the lower phosphorus concentration limit at 0.02 mg. per liter. For the upper limit, system-

atic studies were not made above 0.2 mg. per liter, although calibration corrections for 0.4 mg. per liter were determined occasionally and found to be about twice as large as those for 0.2 mg. per liter.

Effect of pH Differences—The color intensity was strongly influenced by the pH of the sample-reagent mixture. Although the pH value of the mix-

TABLE V

Interfering Substances

Tolerance = the molarity corresponding to 0.5 per cent deviation of the extinction reading for 0.1 mg. per liter of P. The tolerance ratio = the tolerance divided by the molarity of phosphate.

Substance	Change in extinction	Tolerance	Tolerance ratio
		moles per l.	
Urea	Not observed	$>0\ 01$	>3000
NaCl	Decrease	0.005	1700
CaCl$_2$	"	$0\ 003$	1000
Mg^{++} (added as MgSO$_4$)	Increase	$0\ 0012$	400
Glucose	"	0.0010	300
NaF	Decrease	0.0005	170
Lactic acid	"	$0\ 0002$	70
Fe^{+++}, added as FeNH$_4$(SO$_4$)$_2$	Increase	$0\ 00005$	17
Na$_2$SiO$_3$	"	0.00002	7
Citric acid	Decrease	0.000003	1

ture mainly depended upon the added molybdate solution, the acid content of the sample was not without effect on the extinction reading. The following observations were made with the phosphate standards.

	pH changed by doubling content of sample in	
	H$_2$SO$_4$	CCl$_3$COOH
pH change in phosphate sample	0.26	0.35
" " " color mixture	(0.01)*	0.03
Decrease in extinction, %	6.3	3 4

* Calculated.

The above pH differences are much greater than would be expected from volumetric errors and variations in alkalinity of the tissues and sera. The observed pH differences between the standard and unknown solutions were not over 0.02 unit. Hence, the resulting errors in the extinction values probably did not exceed 0.5 per cent.

The greatest inconvenience caused by pH effects was in the use of photometer cells with fused on windows (*cf.* p. 447).

Interfering Substances—Protective colloids, for instance proteins and

peptones, retarded the development of color and decreased its intensity, although the amounts remaining in serum filtrates apparently did not interfere (*cf.* Table I). The presence of CCl₃COOH in the filtrates increased the readings by about 10 per cent. To compensate for this, the phosphate standard contained an equal amount of the acid plus sufficient Na₂CO₃ to match the pH values of the filtrates.

Some constituents of calcified tissues and serum filtrates and substances known to interfere in the molybdenum blue methods were tested for the effect on readings by the quinaldine red method, in 0.01 M or lower concentrations. The observed tolerance levels (*cf.* Table V) were above the concentrations normally present in such samples, with the possible exception of citric acid in serum.

The tolerance level of citric acid was increased to 0.00001 M by making the molybdate-sulfate solution 0.4 M in H₃BO₃.

Estimates of Accuracy; Checks against Molybdenum Blue Method

A modification of Mueller's amidol method (5) compared favorably with other molybdenum blue methods which were tested in that the readings were highly reproducible and stable and the effects of temperature and pH were small. The use of amidol as the reducing agent has been recommended by Allen (6) and others. Because of the lower sensitivity of the amidol method, undiluted serum filtrates were sampled, while the dissolved tissue samples were 20 times more concentrated than in the quinaldine red method.

In the analyses by the quinaldine red method, 2 ml. samples of the calcified tissue solutions and diluted serum filtrates were processed and read in 14.5 mm. tubes. Three samples of the unknowns and three to six samples of the standards were taken.

The findings are presented in Table I. The divergence between the quinaldine red and molybdenum blue values averaged 1.0 per cent for the calcified tissue samples and 0.65 per cent for the serum unknowns. The differences can be accounted for by the previously discussed sources of error, errors of color measurement, and effects of pH differences and variations in the amount of molybdate-sulfate in particular. The molybdenum blue values, it should be noted, were also subject to an uncertainty of the order of 0.5 per cent.

Differences averaging 6 per cent have been reported when two molybdenum blue methods were compared (7), one of which (1) was sensitive to pH and other chemical factors and the other (2) relatively insensitive.

METABOLISM OF STEROIDS BY TISSUES

I. DETERMINATION OF TESTOSTERONE AND RELATED STEROIDS IN TISSUE EXTRACTS*

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Since the steroid hormones are formed in, act upon, and are metabolized by animal tissues, methods by which these compounds could be determined in tissue extracts would enable the investigator to study their metabolism. By the method of preparation of tissue extracts to be described, testosterone, androsterone, and other neutral keto steroids can be recovered quantitatively in sufficient purity to enable various colorimetric and spectrophotometric methods to be applied. In our laboratory the method has been particularly applied to tissues incubated with added hormone, and the description of procedure is based on such a preparation. By suitable modification of the preliminary procedure, the method has been applied to blood and perfusing fluids. The three essential steps are (*a*) the removal of most of the protein, (*b*) chromatographic adsorption, (*c*) distribution between immiscible solvents.

Method

The method as finally developed is as follows. The minced tissue in the incubating fluid is boiled under a reflux for 20 minutes to destroy all enzymic action and to coagulate protein. The flask containing the mixture is cooled in a cold water bath while still attached to the condenser, and the condenser is washed down with a small amount of ethanol. This is followed by a larger amount of ethyl ether. When the volume of the original mixture is 25 cc., 2 cc. of ethanol and 15 cc. of ether have proved satisfactory.

The aqueous layer is now thoroughly extracted with ether. The ordinary continuous liquid extractors have not proved satisfactory because the tissue mass is not broken up. Instead we have used a discontinuous method. The aqueous-ether mixture is poured quantitatively into a centrifuge tube, the capacity of which is at least 3 times the volume of the original aqueous suspension. For the 25 cc. portions already mentioned 80 to 100 cc. centrifuge tubes are suitable. The ether, tissue, and water are then thoroughly

mixed together by a stirring motor or by a rapid up and down motion of a stirrer for 2 minutes.

After stirring, the tubes are centrifuged to break the emulsion, and the ether layer is blown off into an Erlenmeyer flask with a ground glass joint. The extraction is then repeated four additional times.

The combined ether extracts are evaporated to dryness in the glass-jointed flask. To the residue 20 cc. of redistilled pentane (Skellyrolve A) are now added and the flask is stoppered with a glass stopper and allowed to stand 4 hours at room temperature or overnight in the refrigerator. This standing is important, as the hormones have only a low solubility in pentane and dissolve slowly.

A chromatographic column is now prepared with Baker and Adamson's aluminum oxide, reagent grade ignited. Merck's aluminum oxide, according to Brockmann, is too retentive. The column of aluminum oxide is 10 mm. in diameter and 50 mm. long. It is packed by gentle tapping while being filled. A small plug of Corning glass wool is used at the bottom of the column.

The following solutions are then passed through the column and collected as indicated, care being taken not to let any air enter the column between fractions: 10 cc. of pentane, 20 cc. of pentane solution of sample, 30 cc. of pentane, 15 cc. of 10 per cent $CHCl_3$ in pentane, 75 cc. of 25 per cent $CHCl_3$ in pentane, 50 cc. of $CHCl_3$, collected as the discard, Fraction A, Fraction A, Fraction B, Fraction C, Fraction D, respectively. Fraction C contains the hormones mentioned, but Fractions B and D are usually checked if any new tissue or hormone is being studied or a new batch of aluminum oxide is being tested.

Fraction C is now evaporated to dryness, redissolved, and rinsed into a separatory funnel with 20 cc. of pentane or petroleum ether and 20 cc. of 70 per cent ethanol in water. The two layers are shaken together and the alcohol layer is drawn off and saved. The pentane layer is again extracted with 15 cc. of 70 per cent ethanol and the ethanol layer combined with the previous portion (1). The pentane is now discarded.

The combined 70 per cent ethanol layers are now transferred to a separatory funnel; 15 cc. of water are added, and the solution extracted with three 15 cc. portions of $CHCl_3$. The chloroform extract is then evaporated to dryness. This step can be eliminated if it is more convenient to evaporate the 70 per cent ethanol solution to dryness at 50° under a vacuum. The ethanol and water cannot be evaporated on the steam bath at atmospheric pressure, however, without loss of some testosterone.

The material so prepared usually is colorless and semi-crystalline. It can be dissolved in ethanol or other suitable solvent and used in methods involving concentrated sulfuric acid such as the Koenig and Samuels (2)

method for testosterone, or, with strong base, as in the modified Zimmermann reaction (3). It will also give good curves in the ultraviolet region of the spectrum.

DISCUSSION

The over-all recoveries with hormone plus boiled tissue are given in Tables I and II. The tissues were minced and washed into a 125 cc. Erlenmeyer flask in which the hormone was dissolved in 0.15 cc. of ethanol.

TABLE I

Recovery of Various Steroids from Liver Extracts*

Steroid	Amount added	Amount recovered (ultraviolet)†	Amount recovered (colorimetric)
	γ	γ	γ
Testosterone	200	202	201‡
"	200	195	216‡
"	400	386	
"	400	385	
"	400	402	402‡
"	400	398	401‡
Methyltestosterone	200	197	
Progesterone	200	212	
"	200	197	
"	400	395	
Testosterone diethylaminoethyl carbonate hydrochloride	300	289	269‡
Mixture of testosterone	200	199	197‡
+ androsterone	400		367§

* Enzymic action of the tissue suspension was stopped by boiling immediately after the introduction of the steroid.

† Based on the absorption band at 238 mμ, due to α,β-unsaturated ketone. Determination made on a Beckman spectrophotometer.

‡ Determined by the colorimetric reaction of Koenig *et al* (2).

§ Determined by the Callow modification of the Zimmermann reaction (3).

Usually 25 cc. of Locke's solution were used to wash in the tissue. The mixture was then brought to a boil to stop enzymic action on the hormone. The extraction was carried out as described.

The greatest problem in applying steroid reactions to tissue extracts is that the other lipides also give colors with the strong reagents used, or absorb in the ultraviolet spectrum. Unsaturated aliphatic compounds are particularly bad. Neither partition between 70 per cent ethanol and pentane nor chromatography alone yielded sufficiently pure extracts, but a combination of the two was effective.

The sequence must be chromatography, followed by partition, rather than vice versa. Attempts to reverse the order always resulted in significant amounts of interfering lipides. The chromatographic column apparently removes some substance which affects the partition; either this or a certain amount of lipide material is held in the column, even when the total fat is lowered by carrying out the partition first, and this is always washed through in subsequent fractions.

Obviously in using any new steroid its distribution in the chromatographic eluates must be determined before the method is applied. The fractions described are sufficiently coarse to yield testosterone, androsterone, pro-

TABLE II

Recovery of Added Testosterone from Extracts of Tissue Boiled in Locke's Solution, Estimation by Koenig Colorimetric Method

Tissue		Weight	Amount recovered (200 γ added)	Recovered
		gm	*γ*	*per cent*
Rabbit liver		1.15	200	100.0
" "		1.17	210	105.0
" uterus		0 71	192	96.0
" "		0.92	215	107.5
Mouse tumor		0.705	196	98.0
" "		0.610	144	72.0
" "		0 626	210	105.0
" liver		0.510	188	94.0
" "		0.420	192	96.0
" "		0.506	206	103.0
Rat liver		1 47	184	92.0
" "		1.28	187	93.5
" "		1 14	201	100.5
" "		0.825	187	93.5

gesterone, and similar compounds in the 25 per cent chloroform fraction. Other steroids may come out in the other fractions, however. One may wish to fractionate the steroids further on the column. This can be done by using smaller increments of chloroform. The procedure used here was best adapted for our particular studies, but a wide range of variations in this step may be used.

SUMMARY

A method of extraction has been described whereby steroid hormones may be isolated from tissues in sufficient purity to permit the application of various steroid reactions involving strong reagents. The basis of the

method is a chromatographic separation followed by partition between 70 per cent ethanol and pentane or petroleum ether.   Recoveries are within the limits of error of the techniques of estimation used.

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METABOLISM OF STEROIDS BY TISSUES

II. THE ENZYMIC DESTRUCTION OF TESTOSTERONE
BY LIVER TISSUE*

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The mechanisms whereby testosterone is inactivated in tissues have been the subject of considerable speculation, but little direct information has been obtained. Advantage was taken of a method of isolation of steroids from tissue extracts (1) together with a relatively specific color reaction (2) and ultraviolet absorption to study the reaction of liver mince with testosterone.

Biskind (3) has demonstrated by implantation experiments that testosterone is inactivated by the liver. In rats suffering from a deficiency of the vitamin B complex, the livers were still able to destroy this hormone but not estrogen (4). Danby (5) reported that male hormone added to blood largely disappeared during perfusion through dog livers or cow kidneys. She did not observe any destruction when testosterone, androsterone, or dehydroandrosterone was incubated with liver or kidney pulp *in vitro*. Here again the destruction of testosterone appears to differ from that of estrogens; Zondek (6) reported that even dried powders and cell-free extracts of liver destroyed the latter. Thus the evidence for enzymic destruction of the androgens is definitely less satisfactory than for the estrogens. In the case of the androgenic hormones the evidence has been based on biological assay and cannot be interpreted chemically.

EXPERIMENTAL

Animals which furnished the tissue for these studies were decapitated with a heavy sharp knife and allowed to bleed freely. The tissues were then removed and quickly minced with an instrument made up of a series of safety razor blades separated by 0.20 mm. dividers. Samples of human liver were obtained by biopsy during abdominal operations and were minced as soon as possible thereafter. The minced tissue was weighed immediately and washed into a flask with the buffer solution to be used. The composition of the buffer solution was as follows: KCl 0.0056 M, MgCl$_2$ 0.0021 M, NaCl 0.08 M, Na$_2$HPO$_4$ + NaH$_2$PO$_4$ buffer, pH 7.4, 0.04 M, glucose 0.1 per cent.

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If an inhibitor was used, an equivalent amount of sodium chloride was eliminated. The solutions were adjusted to pH 7.3 to 7.4.

After the mince was washed in, the flask was immediately closed with a glass stopper and placed in a revolving agitator within a constant temperature bath at 38.5°. 5 minutes were allowed for the tissue suspension to come to temperature equilibrium, incubation time being computed as beginning after this interval. At the end of the incubation the solution in the flask was quickly brought to a boil and refluxed for 20 minutes. The procedure already described (1) was then used for extracting the tissues. The final residue was dissolved in 2.00 cc. of absolute ethanol for analysis.

For determination of testosterone, 0.4 cc. of the alcohol solution was used. The procedure was that described by Koenig et al. (2) with the following modification: a heating time of 6 minutes at 70° was substituted for the first 2 minutes of heating at the temperature of boiling water.

To measure 17-ketosteroids, the Zimmermann reaction was carried out on 0.2 cc. of the alcoholic solution by the method of Callow (7).

The ultraviolet absorption spectrum was determined in absolute ethanol with the Beckman spectrophotometer. Ordinarily 0.4 cc. of the alcoholic solution was used with 3 cc. of ethanol. The absorption band at 238 mμ due to the α,β-unsaturated ketonic group was used for the determination.

To determine whether conjugation was the major route of inactivation, two hydrolytic procedures were used at different times. To some of the early samples 6 cc. of concentrated hydrochloric acid were added before refluxing for 20 minutes. The extraction was then carried out from the 2.3 N acid solution.

In a later experiment all samples were extracted by the regular procedure. To the aqueous suspensions after extraction, however, either sulfuric acid was added to a concentration of 1.4 N or sodium hydroxide to 0.1 N. The solutions were then allowed to stand at room temperature for 16 hours and reextracted. This extract was then carried through the usual analytical procedure.

Results

Effects of Different Tissues—The results of the aerobic incubation of testosterone with minced liver tissue from four different species (rat, rabbit, mouse, and human) are given in Table I. The rate of destruction varied between different livers, but the range was about the same among the different species. The animals used were all adults and apparently in good health. The rats and mice had been on a diet of Purina fox chow for at least 1 month, but their ages varied.

Recoveries from rabbit uterus, rat prostate and seminal vesicle, and mouse mammary carcinoma are also tabulated in Table II. These tissues did not

change the testosterone molecule sufficiently to be measurable by the methods used.

TABLE I

Rate of Destruction of Testosterone by Individual Livers of Different Species

γ per gm. per hr

Rat	Mouse	Rabbit	Human
157	160	171	222 (M.)
121	221	184	236 (40 yrs., M.)
175	127	194	109 (45 " ")
149	159		97 (34 " ")
116	102		125 (34 " F.)
218	297	.	
203	194		
311			

TABLE II

Testosterone Not Significantly Destroyed by Tissues of Secondary Sex Organs

Tissue	Condition	Weight	Testosterone added	Testosterone recovered (Koenig method)	Recovered
		gm	*mg.*	*mg*	*per cent*
Rabbit Uterus 1	Living	0 735	0 200	0 192	96 0
" " 1	"	0.460	0.200	0 190	95 0
" " 1	Boiled	0 410	0 200	0 202	101.0
" " 2	Living	0 44	0 200	0 194	97.0
" " 2	"	1 41	0.200	0 197	98.5
" " 2	Boiled	0 71	0 200	0 192	96.0
" " 2	"	0 92	0 200	0 215	107 5
Rat prostate	Living	0.225	0 300	0 293	97 7
" "	"	0.165	0.300	0 292	97 3
" seminal vesicle	"	0 510	0 300	0 275	91.7
" " "	"	0 570	0 300	0.301	100.3
Mouse mammary tumor	"	0 626	0.200	0 210	105 0
" " "	"	0 893	0.200	0.204	102.0
" " "	Boiled	0.705	0.200	0 196	98 0

The Zimmermann reaction was run on all the samples and was negative.

Evidence of Enzymic Character of Reaction—The reaction between liver mince and testosterone was found to depend on an oxidative enzyme system. Table III illustrates the comparative effect of various inhibitors. An atmosphere of nitrogen, the presence of 0.01 M cyanide, 0.05 M iodoacetate, 0.05 M fluoride, or M/15 malonate all partially inhibited the reaction, as

measured by both analytical methods. Boiling for as little as 30 seconds inhibited the reaction completely. The reaction therefore depends on an energy-yielding oxidative system apparently involving metals, sulfhydryl enzymes, and some portion of the Krebs cycle.

TABLE III

Effect of Enzyme Inhibitors on Ability of Liver to Destroy Testosterone

0.200 mg. of testosterone was added to each sample incubated.

Liver		Inhibitor	Testosterone recovered	Testosterone destroyed	Inhibition
			mg.	*mg. per gm. per hr.*	*per cent*
Rabbit (4)*		None	0.058 (0.050–0.066)	0.190 (0.176–0.218)	0
"	(2)	Boiled	0.207 (0.195–0.218)	0	100
"	(2)	0.05 M iodoacetate	0.166 (0.164–0.167)	0.058 (0.056–0.060)	69
"	(3)	None	0.068 (0.053–0.082)	0.184 (0.164–0.211)	0
"	(3)	Boiled	0.205 (0.202–0.208)	0	100
"	(1)	0.01 M NaCN	0.149	0.084	54
"	(2)	None	0.028 (0.024–0.031)	0.169 (0.162–0.180)	0
"	(2)	Boiled	0.205 (0.200–0.210)	0	100
"	(3)	0.01 M KCN	0.089 (0.081–0.097)	0.081 (0.072–0.087)	53
Rat	(3)	None	0.140 (0.130–0.148)	0.083 (0.074–0.093)	0
"	(1)	Boiled	0.200	0	100
"	(3)	N₂	0.181 (0.166–0.193)	0.026 (0.012–0.042)	69
"	(3)	0.05 M NaF	0.163 (0.156–0.176)	0.051 (0.043–0.064)	39
"	(3)	M/15 Na malonate	0.170 (0.157–0.178)	0.042 (0.037–0.052)	49

* The numbers in parentheses indicate the number of samples incubated.

As illustrated in Table IV, attempts to prepare an active suspension in which cellular organization was largely destroyed were unsuccessful. Disintegration of the tissue in the Waring blendor led to inactivation, even though carried out at 0–5° and incubated subsequently with all phases present.

Evidence against Conjugation—To determine whether a compound was formed similar to androsterone sulfate isolated from human male urine by

Munson, Gallagher, and Koch (8) or sodium pregnanediol glucuronidate, isolated from normal and pregnant women's urine by Venning and Browne (9), hydrolytic procedures were carried out.

When hydrolysis was carried out on the crude material immediately after incubation, there was a loss from all samples; recovery from tissue boiled before enzyme action could take place was 62.5 to 68 per cent of the hormone added. Apparently the compound was partially destroyed by

TABLE IV

Effect of Disintegration in Waring Blendor on Ability of Liver Tissue to Destroy Testosterone

| Liver | Weight | Treatment | Testosterone added | Testosterone recovered | | | Testosterone destroyed | Inhibition |
				Koenig	Ultraviolet absorption spectrum	Average		
	gm.		*mg.*	*mg.*	*mg.*	*mg.*	*mg. per gm. per hr*	*per cent*
Rabbit	1 056	Waring blendor, 5°; under N_2	0.200	0.156	0.155	0.156	0.042	78.3
"	1.056	" "	0.200		0.160		0.038	
" boiled	1.056	" "	0.200		0.204			100
"	0.650	Mince	0.200	0.044	0.082	0.063	0.211	0
"	0.780	"	0.200	0.054	0.070	0 062	0.177	
"	0.940	"	0.200	0.038	0.053	0.045	0.164	
' boiled	0.775	"	0.200	0.201	0.204	0.202		100
" "	0.740	"	0.200	0.234	0.208	0 221		100
Rat	1.00	Waring blendor	0.300	0.302				100
"	1.00	" "	0.300	0.309				
"	0.740	Mince	0.300	0.092			0.281	0
"	1.25	Waring blendor, 5°	0.300	0.212			0.070	74.1
"	1.25	" "	0.300	0.224			0.061	
"	0.313	" "	0.300	0.272			0.089	

boiling in the highly acid medium in the presence of tissue. The difference between living tissues and those boiled before incubation still remained, however, the average recovery from living tissues being only 26 to 42 per cent of added hormone, depending on the amount of tissue used. The evidence, therefore, indicates that conjugation alone probably did not account for the destruction.

To avoid the destruction of unchanged testosterone by the acid medium, a second procedure was adopted. The regular extraction was carried out, and the aqueous layer, including the suspended tissue, was then hydrolyzed

with weak acid or weak base.   This solution was then extracted and analyzed by the regular procedure.   No testosterone, no α,β-unsaturated ketones, and no 17-ketosteroids were recovered.

It seems highly improbable, therefore, that the disappearance of testosterone, as measured by these methods, is the result of conjugation either with detoxifying groups or with proteins.

DISCUSSION

It seems apparent from the data presented that the destruction of testosterone in liver tissue is an enzymic series of reactions involving oxidation.   Whether the direct reaction involving testosterone is oxidative cannot be decided in such a complex system.   Unfortunately, so far we have been unable to carry out the reaction in simple systems such as those used by Graubard and Pincus (10) or Westerfeld (11) with estrogens.   The former workers were unable to inactivate testosterone with laccase under conditions which were effective for estrone.

The reaction may involve a coupled oxidation-reduction; considering the ease with which the α,β-unsaturated ketonic group is reduced, this would seem most probable.   Another possibility is that Ring A may be split by oxidation, yielding dicarboxylic acids.   Certainly it seems unlikely that the hormone is oxidized to any stable, intermediate state which would involve additional double bonds or carbonyl groups; otherwise, one would expect absorption bands in the 220 to 350 mμ region.

The disappearance of the band at 238 mμ, as well as the decrease in the colorimetric reaction, rules out Δ^4-androstenedione-3,17 or cis-testosterone as the final products of the enzymic change, since these would give both reactions.   Neither is androsterone nor any other 17-ketosteroid the stable end-product, since the Zimmermann reaction was negative.   It seems unlikely, therefore, that the major pathway of testosterone metabolism in the liver of the rat, mouse, or rabbit involves the androstenedione-androsterone, etiocholanolone route postulated by many workers from the urinary excretion products following testosterone administration (12). Since androsterone is a significant excretion product when large doses of testosterone are given to rats or rabbits, it may be that this compound represents a metabolic pathway in these animals which is followed when the capacity of the normal system is exceeded.   The other possibility is that androsterone is formed in some other organ.

Recently Szego and Roberts (13) introduced evidence that the major portion of the circulating estrogen was present as a lipoprotein complex. Since they could not obtain estrogenic effects on the uterus of the eviscerated rat (14), they have suggested that the combination of estrogens with protein might take place in the liver and that this complex was the effective

agent. They found that the compound was easily split by partial hydrolysis with 0.1 N NaOH or 4 per cent (1.4 N) H_2SO_4. Since similar treatment of the protein residues with these reagents did not release any compounds of the α,β-unsaturated ketonic type nor any 17-ketosteroids, it does not seem likely that this type of combination accounts for the disappearance of testosterone measured in these experiments. Such a compound, however, may also be formed and broken down again to testosterone during the analytical procedure.

SUMMARY

1. With chemical methods of analysis it has been shown that the livers of the rat, mouse, rabbit, and human destroy testosterone. Rabbit uterus, rat prostate and seminal vesicle, and mouse mammary tumor tissue had no significant effect.

2. The destruction was inhibited by boiling, cyanide, iodoacetate, fluoride, malonate, and an atmosphere of nitrogen. The reaction appears to be an oxidative enzymic one involving metals, sulfhydryl enzymes, and the Krebs cycle. Inhibition was not complete, except by boiling, and the hypothesis was introduced that the usual oxidative systems form some substrate which is directly utilized in the reaction with testosterone.

3. No evidence was found that conjugation was a major factor. Since the absorption band at 238 mμ decreased as the color reaction diminished, it appears that the α,β-unsaturated ketonic linkage was destroyed. Since no new bands appeared in the region of 220 to 350 mμ, carbonyl groups seem to have been destroyed. No evidence of the formation of 17-ketosteroids was found.

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THE PHENYLALANINE AND TYROSINE CONTENTS
OF CHICKS AND EGGS*

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One of the most direct methods of studying the metabolism of such closely related amino acids as phenylalanine and tyrosine is to estimate the amino acid contents of the animal carcass at the start of the experiment, calculate the intakes from feed consumption data, and determine the amino acids present at the end of the experiment. By the use of proper diets, information can thus be obtained concerning the extent of conversion of one amino acid into another and the efficiency of utilization of amino acids under various dietary régimes.

The present report is concerned with the determination of the phenylalanine, tyrosine, and nitrogen contents of chick carcasses at various stages of growth and with certain changes which occur as egg protein is transformed into chick protein.

The data on the amino acid contents of entire animals are sparse and variable. Block and Bolling (1) found 3.5 to 4.3 per cent phenylalanine and 2.9 to 3.6 per cent tyrosine in the crude protein (N × 6.25) of entire rats; age had no effect on the amino acid content. Albanese (2) found 4.8 per cent phenylalanine in the crude protein of mature rats. Calvery (3) studied the changes in the tyrosine content of the egg during incubation, and found that the tyrosine content of embryo protein decreases with age, while that in the yolk and white remains constant. Munks *et al.* (4) found 5.8 per cent phenylalanine and 4.8 per cent tyrosine in the protein of whole eggs.

In the present study, the method of hydrolysis of the whole chick had to be suitable for phenylalanine, tyrosine, and nitrogen analyses; hence, acid hydrolysis was used in order to destroy tryptophane, which is said to interfere with the analyses for tyrosine and phenylalanine. 6 N sulfuric acid was used instead of 6 N hydrochloric acid, because the high level of chloride interferes with the Millon color reaction for tyrosine. Before the chick carcasses could be analyzed, it was found necessary to develop certain modifications of the original Kapeller-Adler and Millon methods for determining phenylalanine and tyrosine, respectively.

Kapeller-Adler Method for Determining Phenylalanine—The original method (5) has been studied and modified by Block *et al.* (6, 1), by Brown (7), by Albánese (2), and by Hess and Sullivan (8). In applying the method

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of Albanese (2), it was found that introduction of the aliquots directly into 50 ml. volumetric flasks eliminated transfer from evaporating dishes, and made it possible to dry a large number of flasks simultaneously. Since more intense color was obtained when flasks were used (rather than evaporating dishes), the sensitivity was increased (Fig. 1, lower curves). The differences in the transmittance curves reflect the slight color differences obtained with the two methods; the violet tinge of the original color was replaced by a reddish brown. Albanese, who used a photoelectric color-
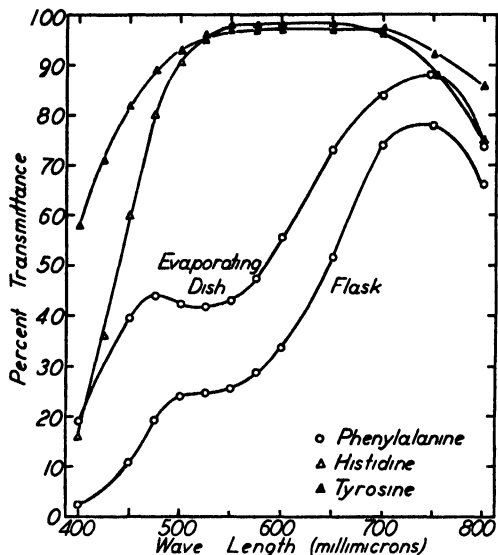
FIG 1. Transmittance curves of the color produced by 3 mg each of phenylalanine tyrosine, and histidine, according to the modified Kapeller-Adler method described in the text. In the two lower curves, a comparison is made between the original (evaporating dish) method and the flask method used in the present study. The histidine and tyrosine colors were produced in flasks. From these data, λ 550 mμ was chosen as the wave-length at which readings were made.

imeter to measure the color, found that, in order to eliminate erroneously high values, tyrosine and histidine had to be destroyed. Tyrosine was destroyed by $KMnO_4$ in acid solution, and histidine was adsorbed when the solution was passed through a column of permutit. Tryptophane presented no problem, because Albanese used an acid hydrolysate. Brown (7) used a spectrophotometer, but since he used an alkaline hydrolysate, tryptophane had to be removed by precipitation as the mercury salt. Brown used an aliquot to which no hydroxylamine was added as a standard, instead of a more reproducible standard such as water. Among the modifications which were introduced in the present study to increase the

accuracy were reduction for 10 minutes at 0°, rapid addition of the base, and color development at room temperature for 50 to 60 minutes.

The following method was finally used in all determinations (which were carried out in triplicate). An aliquot containing 1 to 4 mg. of phenylalanine was pipetted into a 50 ml. volumetric flask containing a boiling chip 1 to 3 mm. in diameter. The flask was placed in an oven at 110–120° until the sample was dry or until a thick syrup was obtained, after which 2 ml. of a solution of potassium nitrate in concentrated sulfuric acid (20 gm. plus 100 ml.) were added, and a small test-tube was inverted over the neck of the flask. Nitration was carried out for 30 minutes at 110–120°. The flask was cooled, 5 ml. of water were added, the vessel was again cooled, and 5 ml. of a solution containing 10 gm. of hydroxylamine hydrochloride and 20 gm. of ammonium sulfate per 100 ml. of solution were added. After 10 minutes in an ice bath, 20 ml. of 20 per cent sodium hydroxide were added rapidly with effective agitation. The flask was cooled in ice water to room temperature, diluted to the mark with water, and allowed to stand at room temperature for 50 to 60 minutes, when the color was measured at λ 550 mμ with a Coleman (model 11) spectrophotometer.

In addition to the transmittance curves for phenylalanine, Fig. 1 contains curves for tyrosine and histidine, the two amino acids which have been reported to interfere with the Kapeller-Adler method. At λ 550 mμ, the transmittance values obtained with tyrosine and histidine are very high; hence these amino acids need not be removed prior to analysis. (Even tryptophane has a transmittance value of 98 per cent at λ 550 mμ, and so it would not interfere if alkaline hydrolysates were used.) The straight line obtained when the transmittance was plotted against the concentration on semilogarithmic paper attested to the adherence to Beer's law.

In the analysis of chick carcass hydrolysates, it was found that decolorization by charcoal without prior neutralization removed large amounts of amino acids. Since neutralization was not required, all determinations were made without decolorization.

Modified Millon Method for Tyrosine—The following method is essentially the same as that used by Folin and Marenzi (9), modified by Block and Bolling (1). Duplicate aliquots of a protein hydrolysate containing 0.5 to 1.0 mg. of tyrosine were pipetted into tubes marked at 40 ml., 20 ml. of mercuric sulfate reagent[1] were added, and the tubes were placed in boiling water for 10 minutes. After being cooled to 30°, 1 ml. of 0.8 per cent sodium nitrite was added, water was added to the mark, and the solution

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[1] 75 gm. of mercuric sulfate are dissolved in a mixture of 150 ml. of concentrated sulfuric acid and 500 ml. of water and made to 1670 ml. with water.

was filtered through dry Whatman No. 42 paper. After 10 minutes the red color was measured at λ 475 mμ with a Coleman spectrophotometer.

No color was obtained when this method was applied to phenylalanine, histidine, or tryptophane.

Analyses of Chick Carcasses—A chick was killed by carbon tetrachloride vapor and its digestive tract was removed. The tract was opened, washed, and put with the remainder of the carcass into a wide mouthed, 500 ml. Erlenmeyer flask. The weight of the bird thus cleaned has been designated the *empty weight.* 200 ml. of 6 N sulfuric acid were added, and the flask was put into an oil bath maintained at about 125°. A cold finger condenser prevented water loss. After 18 to 24 hours, the contents of the flask were diluted with hot water, filtered through wet, fluted filter paper (Whatman No. 12), cooled, and diluted so that 5 ml. aliquots could be used for the phenylalanine analysis and 3 ml. aliquots for the tyrosine determinations. Nitrogen was determined by micro-Kjeldahl analysis of the filtrate. Less than 3 per cent of the total nitrogen was lost on the filter, as determined by Kjeldahl analysis of the residue.

Chicks of several ages were analyzed for phenylalanine, tyrosine, and nitrogen by the methods described above. Groups of four chicks each were killed for analysis every 5 days for 30 days. The yolk sacs of the newly hatched chicks of the 4900 series (Table I) were removed and analyzed separately. The yolk sac membrane was included in this analysis. All chicks were fed a commercial type chick mash from the day of hatching until they were killed; they were not fasted before being killed. The empty weight was found to range between 77 and 91 per cent of the live weight, with a mean value of 87 per cent. In Fig. 2, the individual phenylalanine, tyrosine, and nitrogen weights have been plotted on a double log grid against the empty chick weights. These curves have served as bases for estimating the aromatic amino acid and nitrogen contents of chicks of given weights when normal diets have been fed.

The average phenylalanine and tyrosine contents of chick carcass protein (calculated to 16 per cent nitrogen) were found to be 5.61 and 3.26 per cent, respectively. The value for tyrosine lies within the range of the analyses reported by Block and Bolling (1) for rats, but that for phenylalanine is somewhat higher than the value for rat carcass protein already cited (*cf.* also the data for eggs, below).

Analyses of Eggs—Four eggs including shell and membranes were hydrolyzed and analyzed in the same manner as were the chicks, with the results shown in Table I. The average value for phenylalanine, 455 mg., is somewhat higher than the value of 312 mg. obtained by Munks *et al.* (4) for eggs of the same size, but the tyrosine values are very similar (248 mg. against 258 mg.).

The efficiency of the embryo in using the nutrients available to it may be expressed as a percentage recovery (carcass content × 100 ÷ egg con-

TABLE I

Protein, Phenylalanine, and Tyrosine Contents of Eggs and Newly Hatched Chicks

Egg or chick No.	Weight	Weight of contents, calculated*	Crude protein (N × 6.25)		Phenylalanine		Tyrosine	
	gm.	*gm.*	*gm.*	*per cent of content*	*mg.*	*per cent of protein*	*mg.*	*per cent of protein*
Egg								
379	56.0	50.4	5.95	11.8	413	6.95	237	3.99
668	56.5	49.9	5.75	11.5	441	7.67	240	4.17
748	58.5	52.6	5.99	11.3	467	7.80	255	4.26
1048	58.5	52.6	6.23	11.8	499	8.00	255	4.09
Chick carcass minus yolk sac								
4901	29		3.97	13.7	228	5.75	121	3.04
4902	29		4.03	13.9	208	5.16	124	3.07
4903	29		3.59	12.4	220	6.29	128	3.56
4904	26.5		3.19	12.0	190	5.96	106	3.38
Yolk sac								
4901	5.08		1.32	26.0	99	5.98	49	3.70
4902	5.85		1.28	21.9	105	8.21	51	3.99
4903	4.07		0.87	19.7	60	6.90	31	3.90
4904	7.80		2.48	31.8	148	6.37	67	2.70
Chick carcass plus yolk sac								
4901	34		3 29	15.6	327	6.20	170	3.22
4902	35		5.31	15.2	313	5.89	175	3.30
4903	33		4.39	13.3	286	6.53	159	3.62
4904	34		5.67	16.7	338	5.96	173	3.05
4738	37		5.45	14.7	338	6.20	198	3.64
4739	39		5.88	15.1	339	5 76	194	3.30
4740	35		5.51	15.7	333	6.05	193	3.50
4741	37.5		5 75	15 3	349	6.07	198	3 44

* Total weight × 0.9.

tent), but probably a more adequate measure of efficiency is obtained when the yolk sac contents of the hatched chick are taken into account. Approximately 15 per cent by weight of the newly hatched chick is yolk
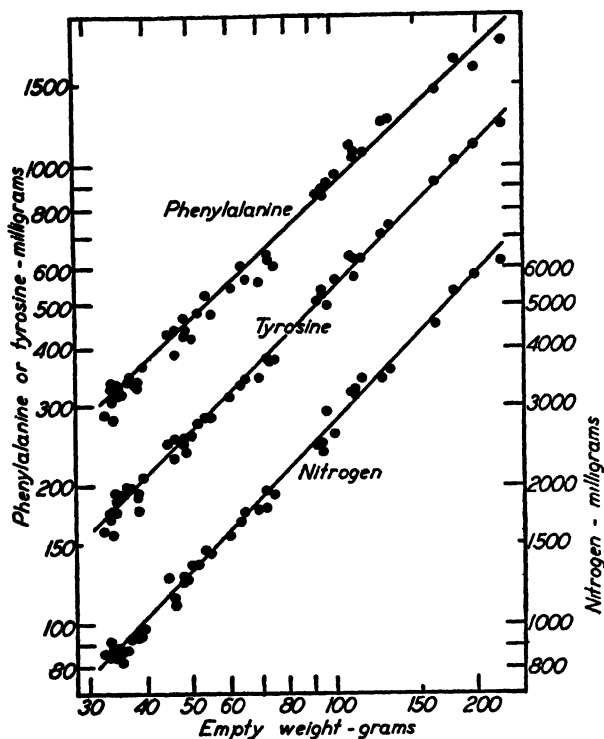
Fig. 2. Individual values of the phenylalanine, tyrosine, and nitrogen contents of normal chick carcasses. The straight lines were drawn by inspection.

TABLE II

Efficiency of Utilization of Protein and Aromatic Amino Acids by Chick Embryo

All chick data corrected to a live weight of 36.5 gm *

	Protein	Phenylalanine	Tyrosine
	gm.	mg	mg
Egg	5 98	455	248
Yolk sac, hatched chick	1.60	111	53
Difference	4.38	344	198
Chick carcass minus yolk sac	3.96	228	129
Recovery by embryo, %	90	66	65

* From the eggs analyzed, chicks would have hatched which would have weighed about 36.5 gm., instead of 34 gm., the actual weight of the chicks analyzed The hatched chick weight is about 64 per cent of the original egg weight, according to data summarized by Needham (11).

material (10) which has not been metabolized,[2] and should not, therefore, be included in the efficiency calculations. From Table II, where the efficiency data calculated in this way are summarized, it is apparent that there is no net interchange of phenylalanine and tyrosine during embryonic development and that the amino acid recovery is lower than that observed with total crude protein. These high efficiencies of utilization of egg amino acids are never approached by the chick after hatching; a 40 per cent recovery of feed amino acids in the carcass is fairly high.[3]

SUMMARY

The Kapeller-Adler and Millon methods for the colorimetric determination of phenylalanine and tyrosine, respectively, were modified and used to determine the contents of these amino acids in chicks of various ages and in whole, unincubated eggs.

The logarithm of the amino acid or nitrogen contents when plotted against the logarithm of the empty carcass weight gives a straight line from hatching to 30 days of age.

Approximately 65 per cent of the phenylalanine and tyrosine present in the egg before incubation is recoverable in the carcass of the hatched chick. 90 per cent of the crude protein (N $\times$ 6.25) is recoverable.

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[2] That the percentages of amino acids in the yolk protein remain unchanged during development was demonstrated by comparison of analytical results for fresh yolk with the data for yolk sac given in Table I. All of the four fresh yolks analyzed contained protein of amino acid content within the range of the yolk sac values.

[3] This feed recovery figure is based on direct data for phenylalanine and tyrosine to be published subsequently as well as on published indirect data of feed consumption and weight gain.

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THE HYDROLYSIS OF PHOSPHOCREATINE AND THE ORIGIN OF URINARY CREATININE

By HENRY BORSOOK and JACOB W. DUBNOFF

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Shaffer (1), Myers and Fine (2), and Hahn and Meyer (3) adduced evidence that muscle creatine is the precursor of urinary creatinine. This was questioned by Chanutin and Kinard (4) but was conclusively proved by Bloch, Schoenheimer, and Rittenberg (5, 6) by isotope tracer evidence, which also showed that creatinine was the only normal urinary constituent containing any significant amount of body creatine nitrogen.[1]

Although Bloch, Schoenheimer, and Rittenberg had settled the issue of the relation between creatine and creatinine, the site and mechanism of creatinine formation and the place of phosphocreatine in the process were still open questions. The crux of all three questions is the fact that the amount of creatinine normally excreted is greater than can be accounted for by the spontaneous (*i.e.*, non-enzymatic) rate of dehydration of creatine at the pH and temperature of the body. Thus Myers and Fine (7) found that the creatine in finely ground muscle was transformed to creatinine more than 3 times as fast as in pure solutions of creatine. Hammett (8) measured the velocity constants of the transformation at 38° and pH 6.9 of the creatine in buffered extracts of rat brain and muscle and in similarly buffered solutions of pure creatine. The velocity constants in the brain suspensions ranged from 0.00083 to 0.00148, average 0.00117; in muscle 0.00091 to 0.00119, average 0.00104; and in creatine solutions 0.00058. (The time unit of these constants is 1 hour.) The percentage of creatine converted in 24 hours calculated from these constants was, in the brain and muscle extracts, 2.8, and in the creatine solution 1.4.

The rate of conversion of creatine to creatinine *in vivo* is of the same order of magnitude as observed by Hammett in brain and in muscle extracts. Table I is an assembly of the data obtained by direct observation on this point.[2]

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[1] Excretion of creatine and creatinine into the intestine and their degradation there were not examined.

[2] Included in Table I are data obtained by Chanutin and Kinard (4), who concluded that, "A statistical analysis of the data obtained in the dog, rat, rabbit, and guinea pig indicates an absence of any relationship between creatinine elimination and the percentage concentration of muscle creatine." We have calculated the correlation coefficient, r, and the value of t (the probability coefficient of r), grouping

TABLE I

Rate of Conversion of Creatine to Creatinine in Vivo

Animal	Average total creatine in body	Average urinary creatinine in 24 hrs.	Creatine converted to creatinine in 24 hrs.	No. of animals	Correlation co-efficient r[*]	t[†]	P[‡]	Bibliographic reference No
	mg.	*mg.*	*per cent*					
Dog	23,020§	477.5	2.41	7	0.951	6.86	<0.01	(4)
Guinea pig	1,197§	27.5	2.66	21	0.880	8.07	<0 01	(4)
Rabbit	3,188	67.7	2.46	10	0.967	10.72	<0 01	(2)
"	6,070§	150.4	2.88	11	0.349	1.12	0.3	(4)
Rat	568§	11.4	2.33	17	0.800	5.6	<0.01	(4)
"	1,026	13 8	1.56	14	0.897	7.04	<0.01	(9)
"			2.03	3				(6)
"			2.70‖					(6)
In vitro from creatine			1.06–1 33					

* Correlation coefficient between the average total body creatine and the average 24 hour urinary creatinine; $r = \dfrac{\Sigma xy}{\sqrt{\Sigma x^2 \cdot \Sigma y^2}}$, where x and y are deviations of individual values of body creatine and 24 hour urinary creatinine, respectively, from their average values for the series.

† $r\sqrt{\dfrac{n-2}{1-r^2}}$.

‡ Probability of obtaining the observed value of r or higher, on the assumption that body creatine and urinary creatinine are unrelated.

§ Estimated as follows: average concentration of muscle creatine × body weight × 0.40.

‖ Calculated from the rate of decrease of isotopic nitrogen in urinary creatinine and in terminal muscle creatine.

in one series all their data on the four animals. The value of r is 0.787 and of t 9.37, which indicates a very high correlation. The statistical treatment of Chanutin and Kinard was inadequate in two respects; (1) they compared the concentration of muscle creatine, which varies little in healthy adult animals with any one species, with the total creatinine elimination, which is affected greatly by the body weight which varies considerably, and (2) the variations within each group of animals of the same species were over too small a range for the number of animals within the group. Table I shows that, when the total body creatine and the urinary creatinine are compared, their data yield high correlation coefficients, except in the case of the rabbit in which again the number of animals was too low for the small range of variation within the group. In the group of rabbits used by Myers and Fine (2) the range of variation was much larger and a high correlation coefficient is obtained. Kinard *et al.* (9) found later in an independent study on the rat a high correlation between total body creatine and creatinine elimination. Any inference in the conclusion of Chanutin and Kinard, quoted above, that body creatine is not related to urinary creatinine was disproved conclusively by Bloch and Schoenheimer (5, 6) by means of creatine labeled with isotopic nitrogen.

The faster rate of conversion of creatine to creatinine in the tissues than, in pure solutions of creatine suggested that an enzyme might be responsible. No evidence of such an enzyme has been found in repeated searches (3, 7, 8, 10). Hahn and Meyer estimated that creatinine elimination in man could be accounted for satisfactorily simply by the spontaneous (*i.e.* non-enzymatic) dehydration of creatine in pure solution, but they used too high a figure for the creatine concentration of human muscle in their estimate, and they took no account of the fact that most of the creatine in the body is not free, but is "bound" as phosphocreatine. Hahn and Meyer's own figure of the rate constant for the spontaneous conversion of creatine to creatinine at pH 7 and 38°, $K = 0.000538$, corresponds to 1.3 per cent conversion in 24 hours, which is lower than the rates observed *in vivo* (Table I), in tissue extracts (7), and in tissue autolysates (8).

In view of the conclusive evidence that the rate of creatinine elimination is related to the amount of body creatine, nearly all of which is in the muscles and in the form of phosphocreatine, we investigated the rate of creatinine formation in buffered aqueous solutions of phosphocreatine and compared it with the rate in similar solutions of creatine. It was found that creatinine is formed spontaneously much faster from phosphocreatine than from creatine. The rate from phosphocreatine at pH 7 and 38° is, in buffered solutions without an enzyme, within the range of rates of transformation of creatine to creatinine observed *in vivo*. It is, therefore, unnecessary to postulate the intervention of an enzyme in this process. That possibility is not excluded, but if there is an enzyme, its activity *in vivo* is low.

<div align="center">EXPERIMENTAL</div>

Phosphocreatine was isolated from rabbit muscle by the method of Fiske and Subbarow (11), and synthesized by the method of Zeile and Fawaz (12). Both preparations were purified in the form of the calcium salt. The same results were obtained with both the natural and synthetic preparations.

Creatine was purified by the method described by Hunter (13).

Creatinine was determined by the alkaline picrate method. We found in solutions containing a large excess of creatine over creatinine that a slow intensification of the color occurs after the initial rapid development. The slow increase in color is, presumably, a consequence of a slow conversion of creatine to creatinine in the alkaline picrate. Accordingly, readings were taken at intervals and the extrapolated zero time value gave the creatinine present before the addition of the alkaline picrate. As an example of the order of magnitude of the extrapolation, the color at the end of an hour

corresponded to 11.5 per cent more creatinine than the extrapolated zero time value.

Inorganic phosphate in the presence of phosphocreatine was determined both by the indirect and by the direct precipitation methods of Fiske and Subbarow. The two methods gave essentially the same hydrolysis constants of phosphocreatine when the room temperature at which the determination was carried out was not too high. We came to prefer the direct method, as the laboratory temperature was often above 25°, and the solutions were warmed somewhat during the colorimetry. Under these conditions the indirect method, as Fiske and Subbarow pointed out, gives values of inorganic phosphate (in the presence of phosphocreatine) which are too high and inconsistent.

The colorimetry of both the creatinine and of the inorganic phosphate was carried out either in a König-Martens (visual) or a Beckman (electrical) spectrophotometer.

0.1 M maleic acid-sodium hydroxide buffers were used.

The preservative employed, after a number were tried, was 0.1 per cent phenol; it did not interfere with either the creatinine or phosphate determinations.

Mathematical Analysis

Three reactions ensue when phosphocreatine is dissolved in approximately neutral solution and at temperatures near to that of the body.

(A) Phosphocreatine $\rightarrow$ inorganic phosphate + creatinine

(B) Phosphocreatine $\rightarrow$ inorganic phosphate + creatine

(C) Creatine $\rightarrow$ creatinine

On the assumption that the three reactions are of the first order, and with the concentration of phosphocreatine designated as [PC], of inorganic phosphate as [P], of creatinine as [Cn], and of creatine as [Cr], the rate of creatinine formation in reaction (A) is

$$\left(\frac{dCn}{dt}\right)_1 = k_1[PC] \tag{1}$$

and in reaction (C)

$$\left(\frac{dCn}{dt}\right)_2 = k_2[Cr] \tag{2}$$

If the initial concentration of phosphocreatine is designated as a, at any time during the reaction

$$[PC] = a - [P] \tag{3}$$

and

$$[Cr] = [P] - [Cn] \tag{4}$$

The total creatinine formed at any time during the reaction is, from equations (1) and (2),

$$\int (dCn)_1 + \int (dCn)_2 = [Cn] = k_1 \int [P\overset{?}{C}] \, dt + k_2 \int [Cr] \, dt \qquad (5)$$

which may be rewritten according to equations (3) and (4)

$$[Cn] = k_1 \int (a - [P]) \, dt + k_2 \int ([P] - [Cn]) \, dt \qquad (6)$$

If equation (6) is rearranged,

$$k_1 = \frac{[Cn] + k_2 \int [Cn] \, dt - k_2 \int [P] \, dt}{at - \int [P] \, dt} \qquad (7)$$

[Cn] is the concentration of creatinine (total) at time t. k_2 was obtained independently from buffered solutions of creatine. $\int [Cn] \, dt$ was computed by graphical integration of the plot of [Cn] against time. To obtain $\int [P] \, dt$ the rate constant of total inorganic phosphate formation from phosphocreatine was determined first; $\int [P] \, dt$ was then computed by graphical integration of the plot against time of inorganic phosphate liberated.

For convenience of computation, the initial concentration of phosphocreatine was set at 100; [Cn] then became the per cent of phosphocreatine converted to creatinine and [P] the per cent of phosphocreatine hydrolyzed in the sum of reactions (1) and (2).

Results

A test of the validity of equation (7) is the constancy of k_1 at different times during the hydrolysis of phosphocreatine at any given pH and temperature. The same test in another form is the agreement between observed and calculated values of [Cn].

The rate constants of the spontaneous conversion of creatine to creatinine in aqueous solution, k_2 in equation (7), obtained by different authors and ourselves, are collected in Table II. The values we obtained lie between those calculated from the data of Myers and Fine (2) and those reported by Hammett (8) and by Hahn and Meyer (3). The values at 38° at or near the same pH are in fairly good agreement; this is appreciated better when the rate constants are expressed as pK values. Thus Hahn and

TABLE II

Rate Constants of Spontaneous Conversion of Creatine to Creatinine

pH	3.96	4.90	5.86	6 00	6.50	6.90	7.00	7.01	7.00	7.50
Temperature, °C.	38	38	38	38	38	38	36	38	38	38
Rate constant	0 00711	0 00207	0 000907	0.000606	0.000507	0 000525*	0.00021† 0.00030	0 000538	0 000442	0.000412
Bibliographic reference No.	(3)	(3)	(3)	Authors	Authors	(8)	(2)	(3)	Authors	Authors

* Hammett (8) gave k from his data as 0.00058; but the small amount of creatinine, which his figures show was present initially in the creatine used, was not taken into account. The value of k above is obtained when this is done.

† The values of k ascribed to Myers and Fine (2) were calculated from their data.

Meyer give, at pH 7.01, pK$_2$ 3.27; we found, at pH 7.00, pK$_2$ 3.35. Neither Hammett nor Hahn and Meyer mention the necessity of extrapolating to zero time when determining creatinine in the,presence of a great excess, 30 to 50 times, of creatine. Our having done so might account for half the difference between their values and ours. Probably the main reason for the difference lies in the fact that the precision of the alkaline picrate method for determining creatinine in low concentrations (0.2 to 1.0 mg. per cent) is not better than ±5 per cent in the lower portion of this range and ±2.5 per cent in the higher portion. The precision was lowered further in all the determinations of k_2 by the great excess of creatine present. The above differences in the values of k_2 at any one pH have a negligible influence on the value of k_1 in equation (7) and may, therefore, be taken as sufficiently concordant for this purpose.

To compute $\int$ [P] dt in equation (7), it is necessary to obtain the rate constant of the liberation of inorganic phosphate from phosphocreatine in the sum of reactions (A) and (B).[3] Table III contains the value of k_P obtained at 20.5° and 38° over a pH range from 5.4 to 7.5. Natural and synthetic phosphocreatine gave the same values (within the experimental error) of k_P at the same pH and temperature. The plot of pk_P against pH, including in one array the values of both natural and synthetic phos-

[3] This constant, k_P, is equal to the sum of the constants of the liberation of inorganic phosphate in reactions (1) and (2), where k_{P_A} and k_{P_B} respectively are the rate constants for reactions (1) and (2). The proof is as follows: The rate of liberation of phosphate in reaction (1) is

$$\left(\frac{dP}{dt}\right)_A = k_{P_A}(a - [P])$$ (8)

and in reaction (2)

$$\left(\frac{dP}{dt}\right)_B = k_{P_B}(a - [P])$$ (9)

The rate of liberation of phosphate in the sum of reactions (1) and (2) is

$$\left(\frac{dP}{dt}\right)_A + \left(\frac{dP}{dt}\right)_B = (k_{P_A} + k_{P_B})(a - [P])$$ (10)

Equation (10) may be written

$$\frac{dP}{dt} = k_P(a - [P])$$ (11)

and since

$$\left(\frac{dP}{dt}\right)_A + \left(\frac{dP}{dt}\right)_B = \frac{dP}{dt}$$ (12)

$$\therefore k_P = k_{P_A} + k_{P_B}$$ (13)

phocreatine, gives at 20.5° a straight line from pH 5.4 to 7.0, and at 38° a straight line from pH 5.8 to 6.5 and a shallow curve from pH 6.5 to 7.5. In Table IV the values of pk_P interpolated from the curves are compared with the observed values at the same pH. There is no consistent trend of disagreement between the values obtained from natural and synthetic phosphocreatine and the corresponding interpolated values. The two preparations may be considered as identical by this criterion.

TABLE III

Hydrolysis Constants of Liberation of Inorganic Phosphate from Phosphocreatine in Buffered Aqueous Solutions

Temperature, 20.5°			Temperature, 38°		
Source of phosphocreatine	pH	Hydrolysis constant	Source of phosphocreatine	pH	Hydrolysis constant
Natural	5 4	0 0298*	Natural	5.80	0.103
"	5.8	0.0109*	Synthetic	6 00	0 0634
Synthetic	6.0	0.00711	Natural	6 43	0.0272
"	6 21	0.00439	Synthetic	6 50	0 0233
Natural	6.4	0 00283*	"	6.70	0.0149
Synthetic	6.5	0.00290	Natural	6.96	0.0101
"	6 55	0.00252	Synthetic	6.96	0.0103
"	6.7	0.00139	"	7 00	0 0101
"	7 02	0 00057	"	7.20	0 00686
			Natural	7 50	0.00553

* Calculated from Fiske and Subbarow's value at 22° (11), multiplied by 2.303 (since their values are for the expression, $k = (1/t) \log (a/(a - x))$ instead of $k = (1/t)\ln(a/(a - x))$ and modified for the difference in temperature between their and our determinations   The equation used to obtain the temperature coefficient is

$$\log\frac{k_2}{k_1} = \frac{E}{2.303R}\left(\frac{T_2-T_1}{T_1T_2}\right)$$

where k_1 and k_2 are the velocity constants at temperatures T_1 and T_2 (on the absolute scale) respectively, R is the gas constant (1.989), and E is the energy of activation. E, calculated from interpolated points on smooth curves, of the rate constants obtained at 20.5° and 38°, is here 23,170 calories. When T_1 and T_2 are 20.5° and 22° respectively, $\log k_1 = \log k_2 - 0.087$.

Table V summarizes the data on creatinine formation in solutions of phosphocreatine at different hydrogen ion concentrations. The initial concentration of phosphocreatine was, in every case, 1.43×10^{-3} molal. Creatinine formed, expressed as equivalent per cent of initial phosphocreatine, was plotted against time, and $\int [Cn]\,dt$ was computed graphically from a smooth plot through the experimental points. In determining the plot, greater weight was given the points at the end of the run than at the beginning. $\int [P]\,dt$ was computed by graphical integration of

the curve of the liberation of inorganic phosphate given by the values of k_P at each pH. The values of k_P and of k_2 used were obtained by interpolation of smooth plots of pk_P and of pk_2 against pH.

The calculated values of creatinine formed were interpolated from curves given by the calculated values of k_1, which, in every case, were near to the mean of the observed values of k_1. The differences between the observed and calculated values of creatinine formed are within the experimental error of the creatinine determination. There was no consistent difference in this respect between the natural and synthetic phosphocreatine. By this criterion, therefore, as well as by the identity of their k_P values, the two preparations behaved the same.

TABLE IV

Comparison of Hydrolysis Constants of Liberation of Inorganic Phosphate of Natural and Synthetic Phosphocreatine

	Temperature, 20.5°			Temperature, 38°			
Source of phosphocreatine	pH	pK observed	Inter-polated value on smooth curve, pK	Source of phosphocreatine	pH	pK observed	Inter-polated value on smooth curve, pK
Natural	5.4	1.53*	1.51	Natural	5.80	0.99	0 95
"	5 8	1.96*	1.93	Synthetic	6 00	1 20	1.18
Synthetic	6.0	2 15	2 14	Natural	6 43	1.57	1 59
"	6 21	2.36	2 36	Synthetic	6 50	1 63	1 66
Natural	6 4	2 55*	2 56	"	6.70	1 83	1 82
Synthetic	6 5	2.54	2.67	Natural	6 96	2 00	2.00
"	6 55	2 60	2 72	Synthetic	6.96	1 99	2.00
"	6 7	2 86	2.87	"	7 00	2.00	2 03
"	7 02	3.24	3 21	"	7 20	2 16	2.17
				Natural	7 50	2 26	2.33

* From Fiske and Subbarow's data; see the footnote to Table III.

Equation (7) and the numerical value of the rate constant k_1 are based on the hypothesis that phosphocreatine is hydrolyzed in part to creatinine and inorganic phosphate and in part to creatine and inorganic phosphate, and the creatine thus formed is independently dehydrated to creatinine. This process is formulated in reactions (A), (B), and (C) above. It is implied that the prevailing concentrations of creatine and creatinine are so far from their equilibrium ratio that reactions (A), (B), and (C) may be considered as irreversible and of the first order.

In this hypothesis the rate of formation of creatinine directly from phosphocreatine is

$$\int d[\text{Cn}_i] = k_1 \int (a - [\text{P}]) \, dt \qquad (14)$$

where [Cn$_1$] is the creatinine coming directly from phosphocreatine, k_1 is the rate constant of that process, a is the initial concentration of phosphocreatine, and [P] the concentration of total inorganic phosphate liberated.

TABLE V

Creatinine from Phosphocreatine at 38°; Creatinine Expressed As Equivalent Per Cent of Initial Phosphocreatine

Initial concentration of phosphocreatine in every case was 1.43×10^{-3} molal, corresponding to 18.7 mg. per cent of creatine or 16.2 mg. per cent of creatinine. Equation (7) and the values of k_P and k_2 at each pH were used for the calculation of k_1. (N) designates that natural and (S) that synthetic phosphocreatine was used.

Time	Creatinine		k_1	
	Observed per cent	Calculated per cent	Observed	Calculated
pH 6.0; $k_P = 0.0634$; $k_2 = 0.000606$				
hrs.				
4.2	1.1 (N)	1.0	0.0029	0.0026
19.4	3.4 (S)	3.4	0.0026	0 0026
24.1	3.85 (N)	3.95	0 0025	0.0025
46.9	6.1 (S)	5.8	0.0029	0.0027
67.6	7.0 "	7.1	0 0026	0 0027
71.5	6.8 (N)	7.3	0 0023	0.0026
Mean	. .	.	0.0026	0.0026
pH 6.44; $k_P = 0.027$; $k_2 = 0.00051$				
19.3	2.6 (S)	2.3	0.0016	0 0014
24.2	3.0 (N)	2.8	0.0015	0.0014
48.1	4.7 "	4.8	0 0014	0 0014
67.5	6.7 (S)	6 1	0.0016	0.0014
71.6	6.4 (N)	6.4	0.0014	0.0014
92.7	7.6 (S)	7.5	0.0014	0.0014
95.8	7.3 (N)	7.7	0.0013	0.0014
Mean..		......	.	0 0015	0.0014
pH 6.91; $k_P = 0.00983$; $k_2 = 0.00045$				
20.0	2.3 (S)	2.0	0.00123	0.00106
23.1	1.9 (N)	2.3	0.00087	0.00106
44.4	4.3 (S)	4.2	0.00110	0.00107
47.0	3.8 (N)	4.4	0 00091	0.00107
69.0	5.4 (S)	6.1	0.00093	0 00107
70.8	5.6 (N)	6.2	0.00094	0.00106
91.3	7.1 (S)	7.6	0.00097	0.00106
94.9	7.7 (N)	7.8	0.00103	0.00105
119.0	9.2 (S)	9.3	0 00104	0.00105
Mean.....			0.00100	0.00106

TABLE V—*Concluded*

| Time | Creatinine | | k_1 | |
	Observed per cent	Calculated per cent	Observed	Calculated
pH 7.21; k_P = 0.00686; k_2 = 0.00043				
hrs.				
20.1	2.65 (S)	2.3	●.0014	0.0012
44.4	5.1 "	4.9	0.0013	0.0012
68.9	6.6 "	7.1	0.0011	0.0012
91.3	8.8 "	8.9	0 0012	0.0012
119.1	11.0 "	11.1	0.0012	0.0012
Mean...	. ...		0.0012	0.0012
pH 7.50; k_P = 0.00550; k_2 = 0.00041				
23.2	2.35 (N)	2 45	0 0011	0 0011
47.0	4.8 "	4 8	0.0011	0.0011
71.5	6.95 "	7.0	0.0011	0.0011
95.0	9 0 "	9 0	0.0011	0.0011
Mean	. . ..	.. .	0.0011	0.0011

The same numerical result as from equations (7) and (14) is obtained on the hypothesis that phosphocreatine in solution consists of two forms which are always in equilibrium with each other. One form, a_1, gives rise on hydrolysis to creatinine and inorganic phosphate, the other, a_2, to creatine and inorganic phosphate. Equation (14) is modified to

$$\int d[\mathrm{Cn_1}] = k'_1 \int ([a_1] - [\mathrm{P_1}]) \, dt \tag{15}$$

which the experimental data showed to be equal to

$$\int d[\mathrm{Cn_1}] = k'_1 \int \alpha(a - [\mathrm{P}]) \, dt \tag{16}$$

where α is the fraction of the total phosphocreatine in the form of a_1. From equations (14) and (16)

$$k_1 = k'_1 \alpha \tag{17}$$

α can be evaluated from the values of k_1 and of k'_1 at any one time in the course of the reaction, or by solving for a_1 in equation (15) at two different times during the reaction. Both methods gave the same value of α, which was constant, at any one pH, during the whole time (up to 120 hours) that the reaction was followed. α varied with pH; it was 0.042, 0.051, 0.104, 0.173, and 0.200 at pH values of 6.0, 6.44, 6.91, 7.21, and 7.50 respec-

tively. On the hypothesis as defined above, of two forms of phosphocreatine, a_1 and a_2, in equilibrium the variation of α with pH indicates that hydrogen ion enters into the equilibrium.

The fact that, at any given pH, α is constant throughout the course of the reaction excludes the possibility that the phosphocreatine we used was a mixture, *i.e.* that a_1 and a_2 were not in equilibrium. If that were the case, since k'_2 (the rate constant for the hydrolysis of phosphocreatine to creatine and inorganic phosphate) is larger than k'_1, α would have varied throughout the reaction. The experimental data showed that this was not the case.

The possibility that the phosphocreatine we used was a mixture is excluded also by the following additional evidence. If it were a mixture,

TABLE VI

Rate Constants (Interpolated) at 38° of Liberation of Inorganic Phosphate from Phosphocreatine, of Creatinine Directly from Phosphocreatine, and of Creatinine from Creatine over pH Range 6.0 to 7.5

pH	k_P	k_{P_A}	k_{P_B}	k_1	k_2
6.0	0.063	0.0026	0 060	0.0026	0 00061
6 5	0.022	0 0014	0 021	0.0014	0.00050
7 0	0.0092	0.0011	0.0081	0.0011	0 00044
7 5	0.0055	0 0011	0.0044	0.0011	0 00041

k_P = the rate constant of liberation of total inorganic phosphate.

k_{P_A} = k_1 = the rate constants of liberation of creatinine directly from phosphocreatine and of the accompanying inorganic phosphate in that reaction alone.

k_{P_B} = $k_P - k_{P_A}$ = the rate constant of liberation of inorganic phosphate accompanying the liberation of creatine from phosphocreatine.

k_2 = the rate constant of conversion of creatine to creatinine.

we might expect the proportions of a_1 and a_2 to be different in different preparations of natural and synthetic phosphocreatine, and accordingly their values of k_P and of k_1 to be different. We used three different preparations of synthetic phosphocreatine and one from rabbit muscle. Fiske and Subbarow obtained theirs from cat muscle. All preparations gave the same values of k_P over the whole experimental range of pH, and all of our preparations gave the same values of k_1. In fact, as shown below, even the *in vivo* rates of creatinine formation in a number of different animals are in accord with that of phosphocreatine *in vitro*.

In the present communication we are concerned primarily with the relation between the rate of spontaneous, *i.e.* non-enzymatic, formation of creatinine from phosphocreatine and the rate of creatinine excretion in the urine. For this purpose it is convenient to use equation (7), as all the terms on the right-hand side are obtained without hypothesis;

the constancy of k_1 at any one pH for all values of t sanctions the use of the equation.

In the literature there are no values of the hydrolysis constants of phosphocreatine over the physiological range of hydrogen ion concentration. These are given in Table VI and also those we obtained of the conversion of creatine to creatinine.

DISCUSSION

In the body most of the creatine (98 per cent) is in the skeletal muscles. Its concentration is kept constant within a range of 400 to 600 mg. per cent in different species. Since the concentration of creatine is kept constant, the per cent converted in 24 hours is $kat = k \times 100 \times 24$. If it were all free creatine and no enzymatic action postulated, the amount converted to creatinine in 24 hours at pH 7.0 would be, with the value of k_2 in Table VI, $24 \times 0.00044 \times 100 = 1.06$ per cent. If it were all phosphocreatine, the value would be $24 \times 0.0011 \times 100 = 2.64$ per cent. The latter is within the range of rates of conversion of creatine to creatinine *in vivo* (Table I), whereas the rate of conversion of creatine *in vitro* is definitely below, approximately one-half, that *in vivo*.

Rosengart (14) found that the formation of creatinine was greatly accelerated in minced muscle at 38° in the presence of fluoride, iodoacetate, or HCN, as did Myers and Fine and Hammett in unpoisoned autolyzing muscle extracts. Rosengart tried to explain this fact by a non-hydrolytic dephosphorylation of phosphocreatine; he later accepted the explanation proposed here, *i.e.* the cleavage of phosphate from phosphocreatine in reaction (1) above.

Some of the data presented by Hammett (8) in 1924 on the rate of appearance of creatinine in autolyzing rat muscle extracts afford a direct comparison with the rate we observed in phosphocreatine solutions at the same pH and temperature. At pH 6.9 and 38° Hammett found at 10, 20, and 30 hours incubation the following percentages of the total creatine converted to creatinine: 1.03, 2.02, and 3.06, respectively. A smooth plot through our experimental data at the same pH and temperature gave for the same time intervals 1.04, 2.03, and 2.98 per cent. After 30 hours incubation Hammett's values are higher than we observed; the explanation is very probably an increase in acidity in Hammett's autolysates, as they were not strongly buffered. Hydrolysis of phosphocreatine itself increases the acidity, and a strong buffer is needed to minimize the latter change. Glycolysis and other autolytic processes also contribute to the increasing acidity. Even after 100 hours incubation Hammett's figure is 9.88 per cent of the original total creatine converted to creatinine; ours (without change in pH) is 8.13 per cent. The agreement is remarkable.

Probably most, but not all, of the creatine in muscle exists as phosphocreatine. In Table VII are collected data in the literature on total creatine and phosphocreatine in the resting muscles of a number of vertebrate species. The ratio of creatine as phosphocreatine to total creatine appears to vary from 43 to 80 per cent. The true values are probably in the upper part of the range; some hydrolysis of phosphocreatine is unavoidable during removal and analysis of muscle; Riesser and Hansen (24) claim that values of phosphocreatine in muscle obtained by Fiske and Subbarow's method, as most of those in Table VII were, are too low; their method gave values 180 per cent of that of Fiske and Subbarow.

TABLE VII
Creatine and Phosphocreatine in Muscle

Animal	Creatine	Phosphocreatine, as creatine	Ratio of phosphocreatine creatine to total creatine
	mg. per cent	*mg. per cent*	*per cent*
Cat	500 (13)	215–363 (11)	43–73
Dog	380 (15)	170 (15)	45
Frog			70–74 (16)
"			80 (17)
Human	309–485 (18)		
"	400 (19)	211–239·(20)	44–68
Rabbit	419–510 (21)	193–291 (21)	
"		301 (20)	59–72
Rat	287 (9)		
"	450 (10)		
"	460 (22)	211 (22)	44–74
"	480 (4)		
. "		164–240 (23)	

The figures in parentheses are bibliographic references.

If we assume, as an average figure, that 60 per cent of the creatine in muscle normally is bound as phosphocreatine, then the creatinine excretion in 24 hours, assuming no enzymatic conversion of creatine or of phosphocreatine to creatinine, is $24 \times 100 \ (0.0011 \times 0.6 + 0.00044 \times 0.4) = 2.02$ per cent of the total creatine in the body. This figure is toward the lower end, but within the range of estimates in Table I.

The hydrolysis rate constants used in the foregoing computations were those at pH 7.0. It may be that the normal resting pH of muscle is lower. Dubuisson (25) gives a range of 6.19 to 7.18 in frog muscle. It seems unlikely that the pH is below 6.0, as Rous (26) reported from his observations with indicators. At pH 6.0, assuming 60 per cent of the total creatine as phosphocreatine, 4.3 per cent of the creatine in both forms would be converted spontaneously to creatinine. As this figure is far above any

observed in animals, the normal resting pH of muscle on the average is probably distinctly above pH 6.0. In any event it is clear that the observed rates of creatinine formation from phosphocreatine *in vitro* are sufficient to account for the amounts of creatinine in the urine of normal animals.

The following facts argue against the participation of an enzyme in the immediate process of creatinine formation *in vivo*. The ratio of daily urinary creatinine to total body creatine is nearly the same in different species (Table I). The ratio is close to that observed in autolyzing muscle extracts and to that observed and calculated by means of equation (7) in solutions of phosphocreatine not containing any tissue extract.

The main lines of the formation of creatine in the animal body may be considered as established (27–31). Normally the creatine formed is retained temporarily in the body as phosphocreatine; the end-product of its metabolism is urinary creatinine. The evidence presented above indicates that creatinine is formed directly by a non-enzymatic cleavage of phosphocreatine, presumably wherever the latter substance exists in the tissues, and hence mainly in the skeletal muscle.

This conception of creatine-creatinine metabolism is in accord with nearly all the data in the literature on normal and abnormal creatinine excretion and creatinuria and on the effects of feeding creatine or increased amounts of its physiological precursors in normal and in pathological conditions; it is in accord with the urinary and muscle findings in the different myopathies. Space does not permit presentation of the data and discussion of them here.

A few findings reported in the literature are not in accord. Outstanding among these is the condition in birds. The major fraction of the creatine plus creatinine they excrete is creatine (7, 32). There has been little study of this aspect of the metabolism of birds, and further investigation is needed. There are some features of creatine formation in birds which differ from those in other animals (27).

In some experiments on man after feeding creatine the data reported give more extra creatinine in the urine than could have arisen spontaneously in the time interval if all the creatine had been converted to phosphocreatine, and, of course, much more than could have arisen directly from creatine (33–35). In most short period experiments little or no extra creatinine was excreted after feeding moderate amounts of creatine. An explanation may be found in the experiments recently reported in a note by Fisher (36). When rabbit kidney was perfused with creatine (10 to 30 mg. per cent) and α-phosphoglycerol, large amounts of creatinine appeared in the perfusate. The requirement of α-phosphoglycerol suggests that the creatine was first converted to phosphocreatine. There may be

an enzyme in kidney which accelerates the decomposition of phosphocreatine to creatinine and inorganic phosphate. Lohmann (10) found no evidence of a phosphatase for phosphocreatine in heart or skeletal muscle. It is unlikely that the kidney is the sole or main site of creatinine formation *in vivo*. If it were, extra creatine production or the feeding of creatine would be followed by increased excretion of creatinine, which is rarely the case. Against the hypothesis of kidney as the main site of formation of urinary creatinine also are the observations of Chanutin and Silvette (37) on the rapid accumulation of creatinine in the blood of nephrectomized rats, the rise in blood creatinine in clinical and experimental kidney disease, and the concordance of the observed ratio of urinary creatinine to total body creatine with that calculated by means of equation (7) and the total creatine in the muscles.

Assigning to phosphocreatine the rôle of immediate precursor of urinary creatinine is, of course, only a modification of the view propounded more than 30 years ago by Shaffer (1), Myers (2, 38), and Spriggs (39) that body creatine is the precursor. Our findings are in accord also with the mechanism of the reaction suggested by Lipmann (40).[4]

SUMMARY

1. The following reactions ensue in aqueous solutions of phosphocreatine at 38°; (A) phosphocreatine → creatinine + inorganic phosphate; (B) phosphocreatine → creatine + inorganic phosphate; (C) creatine → creatinine.

2. They are first order reactions and proceed independently of each other.

3. An equation is developed by means of which the rate constants of reactions (A) and (B) can be determined. The rate constant of reaction (C) was determined independently. The values of the rate constants at 38° over the pH range 6.0 to 7.5 are given. Reaction (A) is 3 to 4 times faster than reaction (C).

4. The combined rate constants of reactions (A) and (C) are sufficient, without postulating the intervention of an enzyme, to correlate quantitatively the reported concentrations of phosphocreatine in muscle and the creatinine excretion in all animal species on which data are available, except birds, and the rate of formation of creatinine in autolyzing muscle.

5. The concept of phosphocreatine as the main immediate precursor of

urinary creatinine does not, as it stands, account for the excretion in birds of much more creatine than creatinine, nor for some few instances in man, in which more creatinine has been reported excreted than can be accounted for by postulating its prior transformation to phosphocreatine and subsequent non-enzymatic hydrolysis of the latter.

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STREPTOMYCIN AND DESOXYRIBONUCLEASE IN THE STUDY OF VARIATIONS IN THE PROPERTIES OF A BACTERIAL VIRUS*

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The chemical compositions of T_2 bacteriophage, reproduced in *Escherichia coli* B grown in lactate, glucose, or broth media, have been reported by two laboratories (1–3). The presence of large amounts of desoxyribose nucleic acid (DNA) in this bacterial virus, regardless of the conditions of growth, has been described. This type of nucleic acid also appears to be present in the T_1, T_3, and T_4 bacteriophages.[1] Ultraviolet irradiation of T_2 disrupted the limiting membrane of the particle and liberated non-sedimentable DNA into solution (4). Although most of the DNA is organized within the virus, it has been found that 30 per cent of the total DNA appears to be present at the surface of T_2 grown in lactate media.[2]

Streptomycin contains the diguanido base, streptidine. From theoretical considerations it was anticipated that streptomycin would combine with the phosphorylated nucleic acids to produce polymeric compounds whose size would depend on the combining ratios of the polyvalent base to polyvalent nucleates. This was found to be true, and at some ratios lattice formation continued until precipitates formed.[3] That the diguanido portion of the molecule was not solely responsible for this complex formation is suggested by the inactivity of streptidine itself in producing these lattices.

Since T_2 bacteriophage (T_2-F) contained about 40 per cent of DNA, it was considered of interest to test its precipitability with streptomycin. Complexes of streptomycin and T_2-F precipitated and this suggested a surface orientation of DNA in the virus. In addition, streptomycin nucleates and complexes of virus and streptomycin were dissociated in M NaCl, as were thymus nucleohistone and nucleoprotamines (5).

Concentrates of T_2-F possessed a relatively high viscosity which was specifically reduced by desoxyribonuclease (DNase) and not by ribo-

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[1] S. S. Cohen and T. F. Anderson, unpublished data.

[2] Virus produced by parasitizing *Escherichia coli* B in lactate medium (F) or nutrient broth (N) will be termed T_2-F or T_2-N respectively.

[3] I am indebted to Dr. P. György of this department for suggesting the possibility of this acid-base interaction.

nuclease, trypsin, chymotrypsin, or lysozyme. The specific reduction in viscosity occurred without loss of virus activity. After DNase digestion, 30 per cent of the previously sedimentable DNA of T_2-F was separable from the virus by high speed centrifugation and the DNase-treated virus had become non-precipitable by streptomycin.

It was noted that concentrates of T_2 prepared from broth lysates (T_2-N) had a low viscosity and were not precipitated by streptomycin. This suggested that virus grown in nutrient broth might have lost the external coat of DNA owing to the activity of an enzyme in broth lysates. It has been observed by Hook *et al.* (6) that T_2 grown in a defined glucose medium possesses dimensions which are slightly but significantly greater than the same strain of T_2 grown in broth (T_2-N). The T_2-N preparations were also more stable than the virus grown in the defined medium.

It was found that broth lysates do contain active DNase capable of reducing the viscosity of T_2-F concentrates, whereas this enzyme was not found in lactate lysates. Furthermore, the relatively unstable T_2-F after treatment with DNase became as stable as T_2-N.

Thus T_2-F appeared to have an outer layer of DNA removable by DNase. On the other hand, the same strain of virus, T_2-N, devoid of this outer layer, appeared to have been already degraded by DNase as a result of growth in a host elaborating this active enzyme in nutrient broth. Variations in the composition of the medium had thus produced variations in the chemical composition, surface structure, stability, reactivity with streptomycin, and the viscosity of concentrates of T_2 bacteriophage.

Materials and Methods

Nomenclature—The relationships of the organism and virus used in these studies to the system of other workers have been described previously (1). T_2 appears to be identical with the PC phage used by Jones (7) in her studies of the streptomycin inactivation of bacteriophage.

Media—*Escherichia coli* B was grown in two media. The defined medium (F) contained 10 gm. of sodium lactate, 1 gm. of NH_4Cl, 0.7 gm. of K_2HPO_4, 0.3 gm. of KH_2PO_4, 0.1 gm. of Na_2SO_4, and 0.01 gm. of $MgSO_4$ per liter of distilled water. The nutrient broth contained 8 gm. of Difco nutrient broth and 5 gm. of NaCl per liter of distilled water. Nutrient agar for slants contained 20 gm. of Difco Bacto-agar, 15 gm. of Difco nutrient broth, 5 gm. of NaCl per liter of distilled water. Plating agar contained 15 gm. of agar per liter of broth medium.

Preparation of Bacteria and Lysates—Overnight liquid cultures were made by inoculating the appropriate medium with bacteria from a slant. The inoculated media were aerated at 37° for 18 hours. At the end of this time they contained about 5×10^9 viable bacteria per cc. For every 10 cc. of the appropriate medium, 0.05 cc. of the overnight culture was added and

the mixture was aerated at 37°. The number of viable organisms was correlated with the turbidity of the bacterial suspension as determined in a Klett-Summerson photoelectric colorimeter with a No. 420 filter. Cultures were grown to 10^8 bacteria per cc. determined turbidimetrically, and inoculated with T_2 bacteriophage at a concentration of 10^6 virus particles per cc. The cultures were aerated at 37° overnight. The resulting lysates, containing about 3×10^9 T_2-F or about 7×10^9 T_2-N per cc., were sedimented at 4000 R.P.M. for 1 hour and the supernatant fluids were stored at 4°.

Preparation of Purified Bacteriophage—The preparation of concentrates of T_2 and their properties have been described (1). Some of the preparations of purified T_2 employed in these studies were kindly given to me by Dr. T. F. Anderson of the Johnson Research Foundation of the University of Pennsylvania. Others were prepared by the same method in this laboratory. Virus assay followed the procedure of counting plaques described by Delbrück and Luria (8) with the spreading and layering modification used by Hershey (9). A virus aliquot was introduced into 2.5 cc. of melted agar containing 7 gm. of agar per liter of broth medium at 45°. The contents of the tube were poured on a solid agar plate, permitted to solidify, and the plate was incubated at 37° overnight.

Other virus preparations have been similarly prepared by differential centrifugation of lysates. These include T_7 from nutrient broth (T_7-N), T_4r^+ from lactate media (T_4r^+-F), T_4r^+ from nutrient broth (T_4r^+-N), and T_4r from nutrient broth (T_4r-N). The term r^+ refers to the size of plaque observed in the assay of the wild type of T_4. The absence of plus indicates that we are dealing with the mutant, T_4r, differing from the wild type with respect to the "r" characteristic, namely the size of plaque reflecting the rapidity of lysis. The preparations of T_2 used in these experiments were of the "r$^+$" type.

We are indebted to Dr. W. M. Stanley of the Rockefeller Insitute for purified preparations of tobacco mosaic virus and tomato bushy stunt virus.

Analyses—The diphenylamine procedure (9) and the perchloric acid-tryptophane (10) method were employed for the estimation of DNA. Phosphorus was estimated by the method of King (11). Nitrogen was determined by the Kjeldahl procedure (5).

Viscosimetry—5 cc. aliquots of the appropriate mixtures were examined in Ostwald viscosimeters at 37.0° ± 0.1°.

Substances—Streptomycin (Abbott) of about 50 per cent purity was employed in initial experiments. These studies were repeated and extended with crystalline streptomycin trihydrochloride and streptidine sulfate kindly given to us by Dr. O. Wintersteiner and Dr. J. Dutcher of The Squibb Institute for Medical Research.

Highly polymerized sodium desoxyribose nucleate (NaDN(p)) was

prepared from thymus. Fresh minced thymus glands were extracted with 2 cc. of H_2O per gm. of tissue at 4°. The nucleohistone in the extract was precipitated by adding NaCl until the solution was 0.14 M with respect to this salt. The sediment was collected by centrifugation and dissolved in M NaCl. The residual thymus was extracted overnight at 4° with M NaCl and the viscous extract was pressed through cheese-cloth and precipitated by pouring into 6 volumes of water. The precipitate was redissolved in M NaCl, combined with the saline solution of dissociated nucleohistone (5), and reprecipitated at 0.14 M NaCl. The sediment of reassociated histone and nucleate was redissolved in M NaCl, adjusted to pH 11.0, and mixed in a Waring blendor for 2 minutes with 0.5 volume of $CHCl_3$-caprylic alcohol (8:1). The mixture was sedimented and the aqueous layer removed from the gel interface. The histone denaturation was repeated until the aqueous layer gave a negative biuret reaction. The pH was reduced to 6.5 with HCl, and the viscous solution of sodium desoxyribonucleate was poured into 2 volumes of chilled alcohol. After standing at 4° overnight, the precipitate was removed by filtration with suction and washed with alcohol and ether. The white fibrous material was dried *in vacuo* over P_2O_5. It has been found that deproteinizing in this manner proceeds more readily at pH 11 than at a lower pH.

Depolymerized sodium desoxyribose nucleate (NaDN (d)) was prepared according to Levene and Bass (12). Two depolymerized sodium ribose nucleates were used, the first that of the Schwarz Laboratories, and secondly, material purified according to Kunitz (13). These three materials were alkali-degraded as a result of the method of preparation.

Oxidized cellulose, containing 19.5 per cent carboxyl and 0.3 per cent nitrogen, was obtained from the Tennessee Eastman Corporation. It was dissolved on the addition of 0.5 per cent NaOH to pH 7.0.

We are indebted to Dr. M. McCarty of the Hospital of the Rockefeller Institute for the preparation of pancreatic desoxyribonuclease employed in these studies. This preparation possessed no proteolytic activity at the concentrations employed, as measured by the inability to reduce the viscosity of gelatin at 37°. Unless otherwise indicated, the enzyme was activated by $MgCl_2$ and stabilized by gelatin according to McCarty (14).

The preparations of crystalline ribonuclease, trypsin, and chymotrypsin were kindly given to us by Dr. M. Kunitz of The Rockefeller Institute at Princeton.

<div align="center">EXPERIMENTAL</div>

Formation and Dissociation of Streptomycin Nucleates—The addition of 1.0 per cent streptomycin (Abbott) to neutral solutions of polymerized NaDN resulted in the formation of opalescent solutions or flocculent pre-

cipitates, depending on the amount of streptomycin added. Repetition of this with 0.5 per cent solutions of crystalline streptomycin trihydrochloide produced essentially similar results, showing a narrow region of soluble opalescent complex formation and a wide region of flocculation. Resolution of the floccules on continued addition of streptomycin was not observed. The development of the complexes was followed quantitatively by adding small aliquots of the appropriate streptomycin solution to 3.0
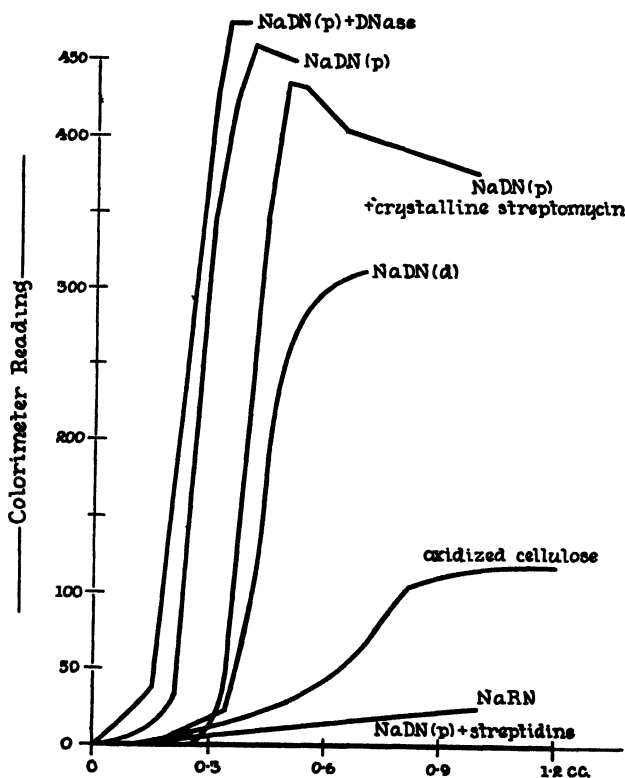
FIG. 1. Complex formation, as measured by turbidity increase, on the addition of streptomycin to solutions of anionic polymers.

cc. aliquots of 0.1 per cent nucleate and examining the opalescent or turbid mixtures in a Klett-Summerson photoelectric colorimeter with a No. 420 filter. In most of the turbidity studies in which relatively large amounts of streptomycin were required, the product of 50 per cent purity was used. The validity of the use of impure streptomycin was established by qualitative studies with smaller volumes of the crystalline trihydrochloride. The results of a number of studies are presented in Fig. 1. Some experiments with crystalline streptomycin and streptidine are included in Fig. 1.

Depolymerization of NaDN with DNase to a viscosity of less than 10 per cent of the original did not reduce the precipitability of the resulting NaDN after 1 hour of depolymerization. Flocculation occurred in the same manner as with NaDN(p). We have observed that treatment of NaDN(p) with this enzyme for 1 hour hydrolyzed only a very small percentage of the phosphate esters to liberate free acid. This suggests that the degraded NaDN employed in this instance still consisted of large molecules whose ability to form lattices was essentially unimpaired.

On the other hand alkali-degraded sodium nucleates such as NaDN(d) and NaRN (Schwarz) have markedly altered turbidity development curves which appear to be a function of the considerably reduced size of the molecules as well as the increased valence per mg. of the smaller polynucleotides. Polycarboxylated compounds, such as the sodium salt of oxidized cellulose, were also capable of reacting with streptomycin, as presented in Fig. 1. All of these compounds were dissociated in M sodium chloride as well as somewhat lower concentrations of salt, as indicated by the total disappearance of opalescence or turbidity.

Streptidine, 1,3-diguanido-2,4,5,6-tetrahydroxycyclohexane, was incapable of forming polymers of this type (*cf.* Fig. 1). This suggests that the weak basic group of streptobiosamine is essential for lattice formation. The addition of streptomycin trihydrochloride to NaDN(p) at pH 11.0 at concentrations for maximal precipitation did not result in precipitation. The addition of weak acid to this mixture resulted in the development of turbidity and precipitation between pH 7 and 8. It has been found by acid titration of streptomycin that the third and weakest basic group binds acid below about pH 9.0 and its titration is at least 80 per cent complete at pH 7.0 (15). Thus one basic group, which participates in and is essential for lattice formation, has a low pK of the order of that in streptobiosamine.

Precipitation of T_2-F Bacteriophage—Ultracentrifugally prepared concentrates of T_2-F in 0.85 per cent NaCl at 10^{10} active particles per cc. and higher were precipitated on the addition of 0.5 per cent streptomycin sulfate. Approximately 40 per cent of the amount of the pure antibiotic was required for maximal precipitation of T_2-F containing a definite amount of DNA, as was required for an equivalent amount of NaDN(p). When the virus contents of the supernatant fluids of different T_2-F preparations were determined immediately after treatment with streptomycin and centrifugation of the precipitate, they represented reductions of 99, 94, 93, 88, 65, and 60 per cent respectively of the initial virus activity. It will be noted in a subsequent section that loss of virus of this magnitude is considerably greater than that observed with T_2-N preparations in the presence of streptomycin for several hours.

The precipitation of T_2-F occurred in thin sheets if solutions of the antibiotic were added slowly to the virus solutions or long fibrous strands when

the mixture was agitated. These precipitates were totally unlike any of the other types of flocculent compounds in appearance. Agitation in M sodium chloride resulted in the dissociation of the precipitates.

Enzymatic Degradation of T_2-F—The formation of streptomycin precipitates with this virus, which contained about 40 per cent NaDN (1), suggested that some of the NaDN was organized at the surface of the particle. It was considered that the interaction of T_2-F, manifested in the relatively high viscosity of concentrates of this material of titer 10^{11} per cc. or higher, might be a function of nucleic acid interaction. The viscosity of T_2-F in the presence of various enzymes was therefore studied.

To 2 cc. aliquots of thrice sedimented T_2-F in 0.85 per cent NaCl at a titer of 5×10^{11} per cc. were added 2 cc. of 0.05 N veronal at pH 7.2 for studies with DNase, ribonuclease, and trypsin, or 2 cc. of 0.05 N acetate at pH 5.0 for the test with lysozyme. To 4 cc. of these mixtures in viscosimeters at 37° were added 1 cc. of 0.0001 per cent DNase, 0.01 per cent ribonuclease, 0.01 per cent trypsin, or 0.01 per cent crystalline lysozyme. The control addition contained 2 cc. of 2.5 per cent gelatin and 130 mg. of $MgCl_2$ per 100 cc. The results presented in Fig. 2 demonstrate that the viscosity of T_2-F concentrates is specifically reduced by desoxyribonuclease.

The various preparations were assayed and were found not to have lost activity. 2 cc. aliquots were sedimented at 60,000g for 1 hour and the supernatants were assayed for activity and tested for DNA. The sediments were resuspended in 0.85 per cent NaCl and tested for streptomycin precipitability. It was observed that, whereas the pellets of DNase-treated T_2-F were readily resuspended, the pellets of the control and other preparations were relatively sticky and difficultly resuspended. These properties of the control and DNase-treated T_2-F are summarized in Table I. The fine turbidity developed on addition of streptomycin to DNase-treated T_2-F is presumably due to reaction with soluble depolymerized DNA which the preparation contains before ultracentrifugation. The sedimented DNase-treated virus did not give this turbidity.

Properties of T_2-N—Concentrates of T_2-N, similarly prepared, having a titer of 10^{11} to 10^{12} active virus particles per cc., possessed a low viscosity, comparable to that of an equivalent titer of DNase-treated T_2. These preparations were not precipitated by 0.5 per cent solutions of streptomycin.

Preparations of T_2-N had a markedly higher content of DNA per active particle than preparations of T_2-F. Since repeated ultracentrifugation of T_2-F is more likely to result in loss of titer than is ultracentrifugation of T_2-N, it might be expected that T_2-F would have a higher relative content of inactive particles and a higher ratio of DNA to activity than did T_2-N. Nevertheless the reverse was consistently observed.

Streptomycin Precipitability of Other Viruses—It was noted that 0.05

cc. of the ribonucleoproteins, tobacco mosaic virus at 10 mg. per cc., and tomato bushy stunt virus at 8 mg. per cc. did not precipitate when mixed
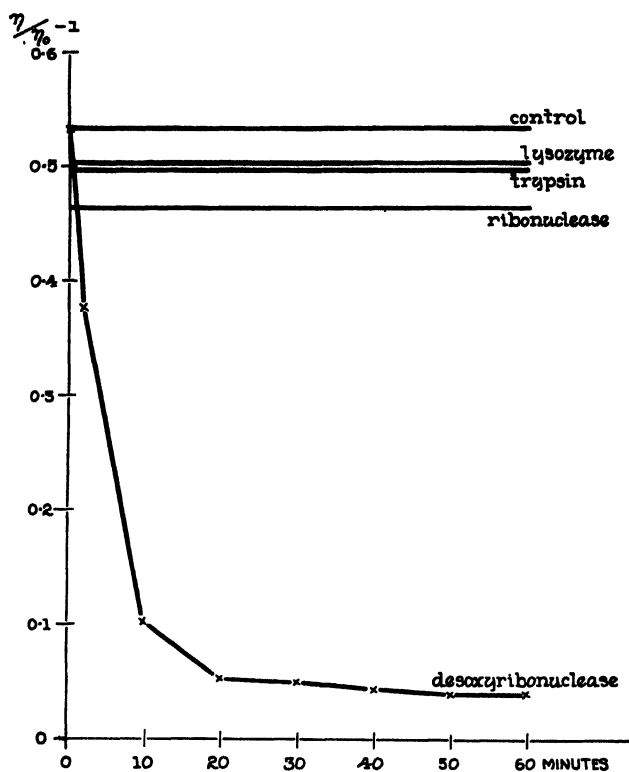
FIG. 2. Action of desoxyribonuclease on a T_2-F concentrate

TABLE I

Properties of T_2-F after Digestion with Desoxyribonuclease

Property	Control	DNase-treated
Specific viscosity at 0.02 mg P per cc.	0 233	0 0167
Streptomycin precipitability of total preparation	Long strands	Fine turbidity
Activity after incubation	$1 98 \times 10^{11}$ per cc	$2 08 \times 10^{11}$ per cc.
DNA per cc. in supernatant fluid after ultracentrifugation	0 029	0 147
% total DNA in supernatant fluid	6 6	33 3
Resuspension of virus sediments	Relatively difficult	Easy
Streptomycin precipitability of re-suspended virus	Long strands	None observed

with 0.1 cc. of 1.0 per cent streptomycin. Under these conditions, T_7-N at 8.8 $\times$ 10^{10} per cc. formed a slight turbidity, as did one preparation of T_4r^+-N at 1.99 $\times$ 10^{11} per cc. Another preparation of T_4r^+-N at 3.65 $\times$ 10^{11} and one of T_4r-N at 3.72 $\times$ 10^{11} gave no precipitate. Preparations of of T_4r^+-F gave the same type of precipitation as T_2-F. In contrast to the low viscosity of T_4r^+-N, preparations of T_4r^+-F were quite viscous. Thus the differences in viscosity and streptomycin precipitability noted between T_2-F and T_2-N appear to exist in the T_4r^+-F and T_4r^+-N preparations as well.

TABLE II

Properties of T_2-F and T_2-N

	F_1	F_2	F_3
Titer of lysate*	2 9 $\times$ 10^9	9.6 $\times$ 10^8	1.7 $\times$ 10^9
" " concentrate	1 05 $\times$ 10^{11}	3 11 $\times$ 10^{10}	6 1 $\times$ 10^{10}
Yield in concentration, %	230	200	220
DNA per cc. concentrate, *mg*	0 075	0 047	0.090
Streptomycin precipitability .	2+	4+	4+
Precipitability after DNase	0	0	0

	N_1	N_2	N_3
Titer of lysate*	5 4 $\times$ 10^9	7 9 $\times$ 10^9	7.6 $\times$ 10^9
" " concentrate	9 9 $\times$ 10^{10}	1 65 $\times$ 10^{11}	1.05 $\times$ 10^{11}
Yield in concentration, %	114	130	90
DNA per cc concentrate, *mg*	0 482	0 644	0.468
Streptomycin precipitability ..	0	0	0
Precipitability after DNase	0	0	0

* After low speed centrifugation to remove débris.

Properties of T_2-F and T_2-N—The previous data were obtained on various virus concentrates that had been prepared at different times. These preparations varied in age, handling, etc. To afford a more careful comparison of the properties of these viruses and the media in which they were grown, three preparations each of T_2-F and T_2-N were made simultaneously as follows: Three 100 cc. aliquots of lactate (F) or nutrient broth (N) were inoculated with *Escherichia coli* B and grown to 10^8 bacteria per cc. Each was inoculated with the same sterile T_2-F concentrate at a concentration of 10^5 per cc. The cultures were aerated at 37° for 18 hours. The lysates were centrifuged at 4000 R.P.M. for 1 hour. The supernatant fluid was assayed for virus and aliquots were removed for estimation of desoxyribonuclease activity. The low speed supernatant fluid was sedimented at 60,000*g* for 1 hour. The pellets were resuspended in 0.85 per cent NaCl in one-sixteenth the volume of the original lysate.

These concentrates were assayed for activity, streptomycin sensitivity before and after desoxyribonuclease digestion, stability alone and in streptomycin, and DNA content.   To 0.8 cc. of the F and N concentrates were added 0.1 cc. of 0.05 N veronal at pH 7.2 and 0.1 cc. of 0.0001 per cent DNase.   The solutions were incubated at 37° for 2 hours.   In Table II
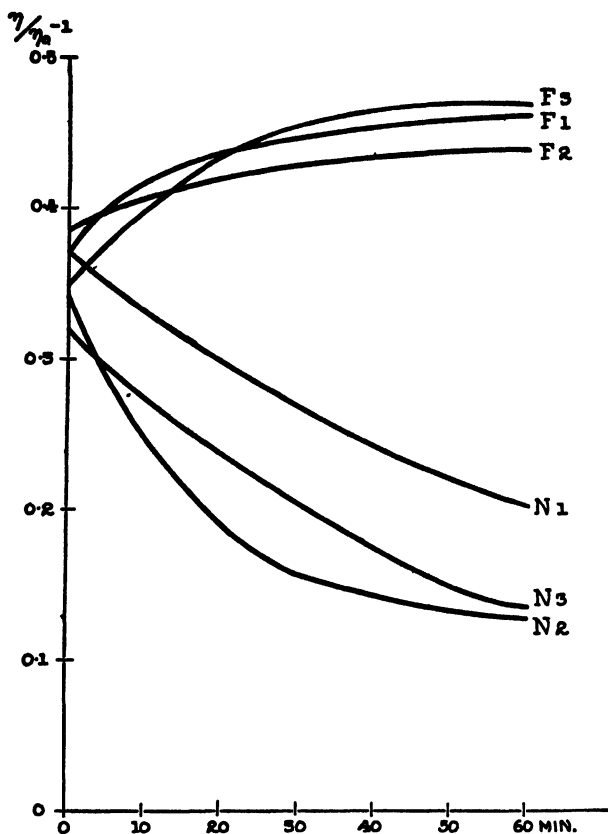
FIG. 3. Depolymerase (DNase) activity of lysates on T_2-F

are presented the titers of the lysates and concentrates, the recovery of activity after sedimentation, streptomycin precipitability, and the DNA content per cc. of concentrate.

From these data it may be seen that the first sedimentation of T_2-F consistently resulted in an apparent doubling of active virus.   This suggests the separation of virus from an inhibitor present in F lysates.   Unpublished data on one-step growth of T_2 on *Escherichia coli* B in the lactate medium have frequently indicated the inactivation of about 30 to 50 per

cent of the virus immediately after maximal lysis. It is possible that an inactive complex of virus and inhibitor was dissociated simply by ultra-centrifugation; this phenomenon is frequently encountered in the ultra-centrifugation of mixtures of neutralizing antibodies and viruses. In addition, the previous observations on T_2-F and T_2-N with respect to strep-tomycin precipitability before and after DNase treatment have been con-
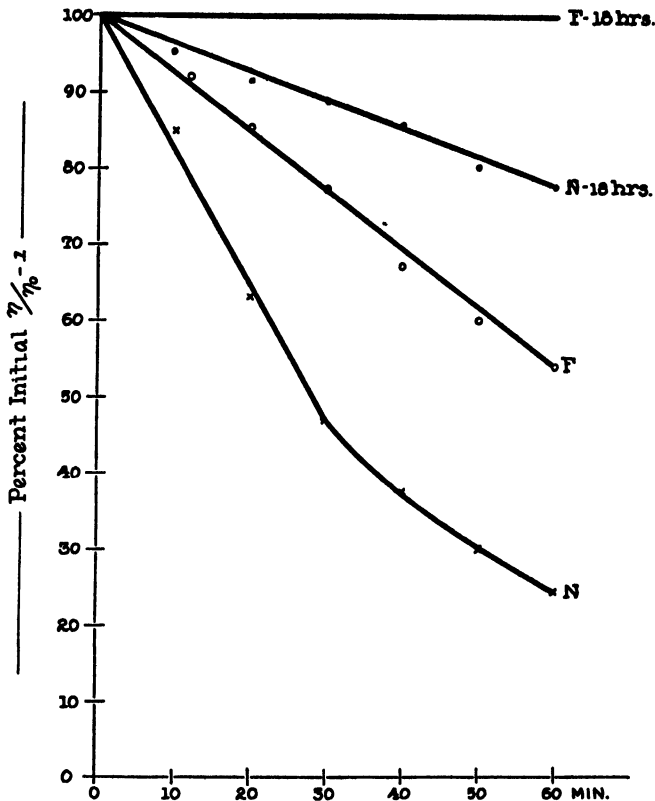
FIG. 4 The activation and stability of pancreatic desoxyribonuclease in lactate medium or nutrient broth.

firmed on simultaneously prepared virus preparations derived from the same inoculum of T_2-F.

DNase Activity of F and N Lysates—To 2 cc. aliquots of T_2-F at 5×10^{11} per cc., previously demonstrated to be specifically reduced in viscosity by DNase only, were added 1 cc. of water and 2 cc. of the appropriate lysate clarified by low speed centrifugation. The viscosity changes of the mix-tures at 37° are presented in Fig. 3.

Activity and Stability of Pancreatic DNase in Different Media—DNase

activity of N lysates over F lysates may be explained in many ways. Two possibilities were tested: (1) that nutrient broth activates DNase to a greater extent than the protein-free and Mg-low F medium; (2) that the stability of DNase is greater at 37° in broth than in lactate. Both conditions were found to be true.
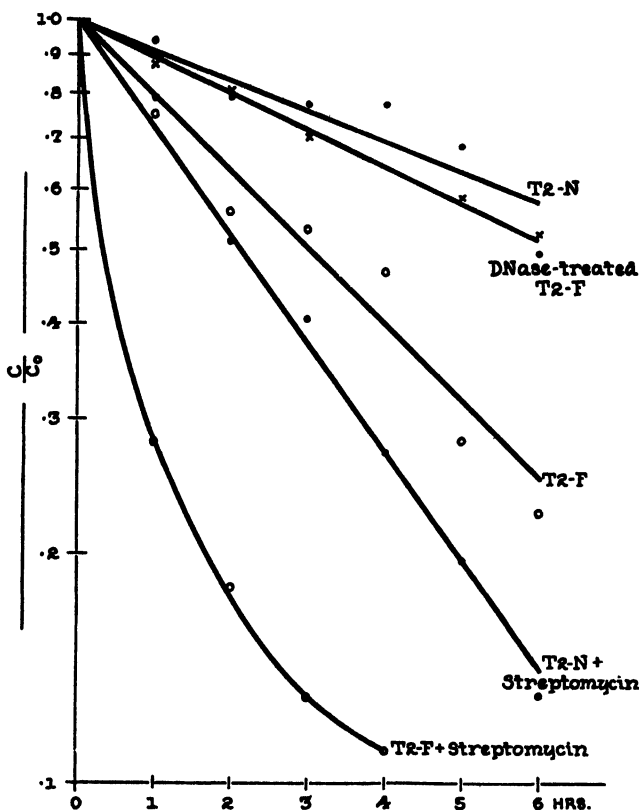
FIG. 5. The inactivation of T_2 at 37°. c/c_0 represents the fraction of residual activity where c_0 is the initial titer and c the titer at the time of assay after incubation.

A 0.01 per cent solution of pancreatic desoxyribonuclease was freshly prepared in the absence of added $MgCl_2$ and gelatin. The enzyme was diluted 1:10 in broth or lactate (F) of the same pH. To 4 cc. of 0.1 per cent NaDN(p) were added 1 cc. aliquots of the diluted enzyme. From Fig. 4 it can be seen that DNase diluted in broth reduced the viscosity of NaDN(p) faster than the same quantity of enzyme diluted in F.

The 0.001 per cent DNA in broth and F were kept at 37° for 18 hours. At the end of that time, 1 cc. aliquots were retested with 4 cc. of substrate.

DNase in F had lost all of its activity in contrast to the DNase added to broth, which still possessed some activity. These data also are presented in Fig. 4.

Effect of DNase on Stability of T_2-F at 37°—The concentrates of T_2 described in Table II were used in these studies. They included N_3, F_3, and DNase-treated F_3. The concentrates in 0.85 per cent NaCl were diluted in saline to about 2×10^8 active particles per cc. To 0.5 cc. of diluted virus was added 0.5 cc. of 0.05 N veronal at pH 7.2. The solutions were incubated at 37° and assayed over a 6 hour period. The inactivation curves are presented in Fig. 5. The inactivation of these materials appears to
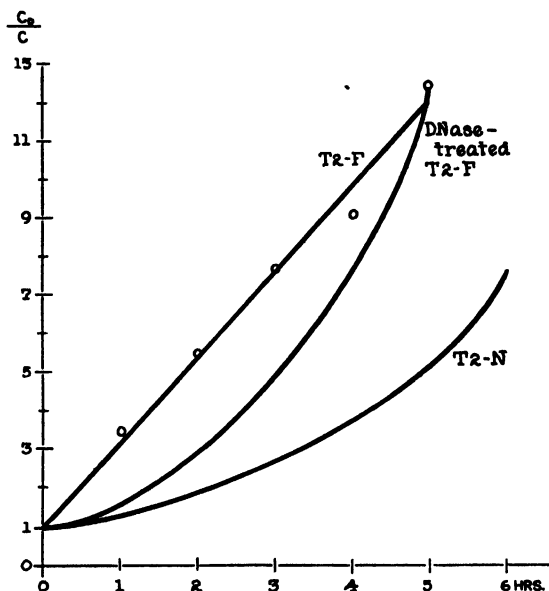
Fig 6 The inactivation of T_2 by streptomycin at 37°

follow a first order reaction; the velocity constant for the inactivation of T_2-F is about twice that of T_2-F treated with DNase and T_2-N.

Streptomycin Inactivation of T_2—To 0.5 cc. of the same preparations of N_3, F_3, and DNase-treated F_3 was added 0.5 cc. of 1.0 per cent crystalline streptomycin trihydrochloride in 0.05 N veronal at pH 7.2. The solutions were incubated at 37° and assayed over a 6 hour period. The inactivation of T_2-N by streptomycin appears to follow a first order reaction, whose velocity constant is about 3.5 times that of T_2-N in the absence of the antibiotic, as is demonstrated in Fig. 5. On the other hand, the inactivation of T_2-F by streptomycin is not a first order reaction (Fig. 5) but appears to be a bimolecular reaction, as established by the linear relation between

c_0/c and time, observable in Fig. 6. Thus the inactivation of T_2-N is first order in the presence or absence of streptomycin, while the inactivation of T_2-F is first order in the absence of, and bimolecular in the presence of, streptomycin. DNase-treated T_2-F is intermediate between T_2-N and T_2-F in the rate of inactivation in the presence of streptomycin but possesses a stability close to that of T_2-N in the absence of the antibiotic. It is possible that on prolonged DNase treatment, T_2-F would behave like T_2-N in all respects.

The data of T_2 inactivation by streptomycin have been plotted without correction for the first order inactivation of T_2 alone. In view of the absence of other data which might permit hypotheses as to the mechanism of streptomycin inactivation, the data are presented in this form.

DISCUSSION

It is a matter of interest that streptidine, which lacks antibiotic activity, is incapable of lattice formation with NaDN(p). This suggests a cis configuration of the guanido groups relative to the plane of the cyclohexane, resulting in a spatial orientation that prevents the two guanido groups in streptidine from linking two separated polymeric nucleic acids. On the other hand, it is probably not a coincidence with respect to the mechanism of antibiotic activity that streptobiosamine is essential to lattice formation, presumably by virtue of the third basic group which it supplies. Whether the mechanism of *in vivo* activity involves the binding of specific nucleic acid molecules essential for multiplication processes in the cell is still to be determined. It is possible that the conversion of the methylamine of streptobiosamine to a guanido group might increase the nucleic acid binding capacity at physiological pH and thereby increase the potency of streptomycin.

Since the DNA on T_2-F which may be removed by DNase is not essential to virus activity, the theoretical objection may be raised that this material is not really part of the virus. Also it may be asked whether this material may not have been adsorbed to the virus after lysis. In regard to the first question, we may say in general that we know of no compound possessing biological activity in which every atom is essential for that activity. In the case of tobacco mosaic virus (16), for instance, a considerable percentage of amino groups may be covered without preventing the synthesis of virus containing free amino groups. This question properly depends on a definition for the word "virus," a term upon whose precise significance agreement has not yet been reached. It may be pointed out that the M protein of streptococcus may be stripped from the surface of the organism by proteolytic enzymes (17) without killing the organism or impairing its ability to synthesize M protein in subsequent generations.

In considering the important question of the nature and origin of the externally oriented DNA, (1) the sedimentation of DNA and virus, (2) the stability of T_2 as a function of DNA, (3) the inactivation of T_2 by streptomycin as a function of the surface DNA, and (4) the different sizes of T_2 in broth and simple medium according to Hook *et al.* (6) strongly suggest a structural rôle of this material in the particle-bearing virus activity. The latter workers have described in addition the gradual decomposition of T_2 concentrates derived from synthetic media, with the liberation of soluble material, apparently containing nucleic acid. However, this decomposition product was not readily sedimentable, although it is possible that some was adsorbed to sedimenting virus. In our preparations of T_2-F, described above, the DNA was almost entirely sedimentable. Thus although the apparent external coat of DNA may be attributed to the adsorption of the decomposition product of T_2-F, this does not seem likely, since non-sedimentable DNA was not found in the fresh and seemingly non-decomposed preparations of T_2-F. Regardless of the origin of · the external coat of DNA on T_2-F, a structural difference between T_2-F and T_2-N appears to exist, since we have observed, in confirmation of Hook *et al.* (6), that T_2-N does not readily liberate DNA, in contrast to T_2-F. Our data suggest that the external coat of DNA is the cause rather than the result of this difference.

Although tobacco mosaic virus appears to have its ribose nucleic acid in long strips at the surface of the particle (18), the nucleic acid does not react with ribonuclease while it is on the virus, nor is it removed from the virus by this or other enzymes (19, 20). Furthermore all procedures known to separate the nucleic acid and protein inactivate the virus. It would appear from the inability of tobacco mosaic virus to form a lattice with streptomycin and the ribonuclease insensitivity that the acid groups of this nucleic acid are oriented internally, in covalent linkage with groups of the protein. Thus the surface orientation of nucleic acid in different viruses may be accomplished in various ways, with more or less firmness and importance for the essential quality of the stimulation of duplication.

In the case of the typhus vaccine, it was demonstrated that the main soluble antigen was a degraded product derived from epidemic rickettsiae by the proteolytic activity of the host (21). A similar relationship with respect to enzymatic degradation during cultivation has been noted in this virus system and it may be expected in general that the biologically active products of host-virus interaction are not solely the completed products of synthetic mechanisms.

SUMMARY

Streptomycin and desoxyribonuclease have been employed to study the surface structure of T_2 bacteriophage as a function of their ability to react with desoxyribose nucleic acid. The desoxyribose nucleic acid content, stability, viscosity, precipitability, and rates of inactivation by streptomycin of T_2 bacteriophage have been found to be a function of the conditions of preparation of the virus. These differences appeared in T_4 preparations as well. Parasitism of *Escherichia coli* B in F medium results in the production of virus coated with DNA. Parasitism in the host in nutrient broth results in virus free of this coat, possibly removed by the DNase liberated from the lysed organism. This DNase is activated and stabilized by nutrient broth.

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STUDIES OF AMINO ACID METABOLISM*

I. BLOOD GLYCINE AND ALANINE AND THEIR RELATIONSHIP TO THE TOTAL AMINO ACIDS IN NORMAL SUBJECTS

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Although the concentration of free α-amino acid nitrogen in human blood has been determined accurately by recently developed procedures (1), measurement of the concentrations of the individual amino acids awaits the establishment of specific methods for their determination.

Glutamine comprises 18 to 25 per cent of the free α-amino N in human plasma (2). In addition, studies (3) in which microbiological assay. methods were used give values for ten other amino acids whose total concentrations account for 76 per cent of the value for the free α-amino acid N reported in the literature (1, 4, 5). From these figures it would seem that approximately all of the free amino acids in plasma can be accounted for, despite the fact that glycine, alanine, cystine, proline, methionine, and serine are not included. That these data are inconsistent is indicated from evidence presented below that glycine plus alanine constitutes 24. per cent of the α-amino N of plasma. This finding also may reflect the fundamental rôle of these amino acids in metabolism, despite the fact that they are considered "non-essential."

Little is known concerning the amino acids in erythrocytes. In a few recorded analyses, the α-amino N content has been shown to be about twice as great as that of plasma (1, 6). When, however, the values are corrected for the high glutathione[1] content of red blood cells, the free α-amino N is reported to be almost the same as that of plasma (8).

The development of specific methods for the determination of glycine and alanine (9, 10) made it desirable to establish the concentrations of these substances in the blood of normal subjects. Data are here presented on the content of alanine and glycine in human whole blood, plasma, and red blood cells under fasting and non-fasting conditions. In addition, alterations in the concentrations of these constituents consequent to the ingestion of glycine are reported.

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[1] Glutathione interferes in the determination of free α-amino nitrogen by the ninhydrin-CO_2 method, since the free α-amino carboxylic group of its glutamic acid reacts with ninhydrin, evolving 1 mole of CO_2 per mole of the peptide (7).

Simultaneous measurements of the free α-amino acid N of blood are included, and also some observations on the excretion of glycine in the urine.

Method

Glycine and alanine were measured by methods previously described (9, 10). Total free amino acid N was analyzed according to the method of Hamilton and Van Slyke (1); picric acid was used as the deproteinizing agent. No allowance was made for the urea content of whole blood, which was presumed to be normal.

Venous blood from laboratory workers, interns, and nurses was drawn into bottles containing 1 drop (desiccated) of 20 per cent potassium oxalate per 5 cc. of blood. For determinations of concentrations during the fasting state, blood was obtained just before breakfast after a 12 hour fast. Blood and plasma were deproteinized within 15 minutes after the blood was obtained, and the protein-free filtrates were stored in the refrigerator until determinations were made (most of them within 24 hours after the withdrawal of blood). The glycine, alanine, and free amino N of the red blood cells were calculated from the values obtained for whole blood and plasma and from the hematocrit which was determined in the routine manner. Almost all of the amino acid determinations were carried out in duplicate and the results were averaged.

The effect of glycine administration was studied in three fasting subjects following the ingestion of 1 gm. of this amino acid[2] per 10 pounds of body weight. The material was given in 240 cc. of water or tomato juice. The values recorded in these experiments are single determinations.

For the measurement of the urinary excretion of glycine, the urine passed during a 24 hour period was collected in bottles containing 10 cc. of toluene and 5 cc. of 10 per cent hydrochloric acid. During this same period the dietary intake was recorded and its protein, carbohydrate, and fat content was calculated.

Results

The concentration of glycine in the blood and plasma of ten fasted males and eight fasted females was strikingly uniform (Table I). The average concentration in blood was 2.02 mg. per cent (standard deviation 0.14), in plasma 1.77 mg. per cent (standard deviation 0.26), and in the red cells 2.36 mg. per cent.

Alanine was present in greater concentrations, mole for mole, than glycine (Table I). The average amount in whole blood, plasma, and cells was the same, essentially 4 mg. per cent. Variation in the level of alanine from

[2] Glycine purchased from the Eastman Kodak Company, Rochester, New York.

individual to individual was somewhat greater than obtained with glycine, and there seemed to be no relationship between the·concentrations of these

TABLE I

Distribution of Glycine and Alanine in Blood of Normal Fasting Subjects

Values expressed in mg. per cent; C_g and P_g indicate conçentration of glycine in erythrocytes and plasma respectively, C_a and P_a alanine concentrations.

Subject		Glycine concentration			$\frac{C_g}{P_g}$	Alanine concentration			$\frac{C_a}{P_a}$	Hematocrit cells
		Blood	Plasma	Cells		Blood	Plasma	Cells		
										per cent
Males	B. A.	1.98	1.75	2.20	1.25					50
	G. G.	2.12	2 15	2.08	0.96					44
					·	3.71	3 66	3.78	1 03	45
		1 93	1 67	2 20	1 32	3 90	2 90	4.90	1 69	50
	R. G.	2 04	2 60	1.64	0.63	5 58	5 62	5.55	0 99	58
	M. B.	2.15	1.98	2.48	1.25	4.33	4 26	4.43	1 04	42
	L. S.	1.90	1.54	2 33	1.51	3 84	3 52	4.22	1.20	46
	B. O.	2.08	1.52	2 77	1 81	2.79	3.01	2.52	0.84	44
	D. H.	2.02	1.85	2 21	1 20	4 16	3.89	4.47	1.15	52
	F. R.	2 20	1 88	2 60	1.38	4 53	4.31	4.80	1 11	45
	C. R.	1.76	1 47	2 06	1.40	4.57	4 80	4 32	0.90	49
	J. H	1 89	1 82	1 95	1 07	3 95	4.31	3.52	0 82	46
Average		2 01	1 84	2 23	1.25	4 14	4.03	4 25	1 08	48
Females	N M	2 06	1 75	2 53	1 45					40
		2 17	1 87	2 58	1 36	3 65	3.02	4 55	1.51	41
	G L	1 81	1 65	2.05	1 24	3 39	3.24	3 60	1.11	40
	I E.	2 09	1 48	2 93	1 98	3 11	3.12	2 97	0 95	42
	L. R.	1 93	1 66	2 30	1 39	3 89	4 12	3.57	0 87	42
	B. S.	2 30	1 55	3.53	2 28	3.77	3.43	4.32	1.29	38
	M. M.					4.12	3 99	4 32	1 08	41
		1 95	1 77	2 22	1 26	4 00	4.20	3.71	0 88	41
	K I					5.11	4 77	5.60	1.17	42
	B. B.					3 28	3.75	2.65	0.71	43
	B. G.	1 81	1.72	1 95	1 14	3 89	4 54	2.95	0.65	41
	J. L.	2.14	1 80	2 62	1 45	4 41	4.81	3 86	0 80	42
Average		2 03	1.69	2.52	1.51	3.87	3.91	3.83	1.00	41
Average of all subjects		2 02	1 77	2.36	1 37	4 00	3.97	4·03	1 04	44
Standard deviation		0.14	0 26	0 40		0 62	0.70	0.83		

amino acids. ' No significant difference in alanine or glycine concentration was noted between males and females.

The concentrations of glycine and alanine recorded above are in good agreement with the observations reported recently by Christensen and Lynch (8). These investigators, employing the same analytical methods (9, 10), give plasma glycine values for five subjects which range from 1.50 to 1.98 mg. per cent (average 1.66) and alanine levels on three subjects of 1.50, 2.42, and 3.94 mg. per cent (average 3.36). Their glycine and alanine determinations in erythrocytes are too few to be compared statistically with ours.

The average concentrations of free α-amino N in the blood, plasma, and erythrocytes of nineteen fasting subjects were 5.86, 3.93, and 8.48 mg. per cent respectively. The variation from individual to individual was con-

TABLE II

*Relation of Glycine and Alanine to Total Free α-Amino Acid Nitrogen in Blood**

Subject	Blood			Plasma			Red cells		
	α-Amino N	Glycine	Alanine	α-Amino N	Glycine	Alanine	α-Amino N	Glycine	Alanine
M. B.	4 64	0 286	0 487	3 28	0 264	0 479	6 52	0 331	0.498
B. O.	4.50	0.277	0 313	3 08	0 203	0 338	6 31	0.369	0 283
F. R.	4 93	0.293	0 509	3.57	0 251	0 484	6 59	0.347	0 539
C. R.	4 86	0 234	0 514	3 31	0 196	0 540	6 46	0 275	0 485
J. H	3 88	0 252	0 444	3 02	0 243	0 484	4 89	0 260	0.396
G. L.	3.44	0 241	0 381	2.48	0 220	0 364	4 89	0 273	0 404
N. M.	4 07	0.260	0 449	2 73	0 236	0 472	6 16	0 296	0 417
B. G.	3 96	0.241	0 437	2 81	0.229	0 510	5 61	0 260	0.331
J. L.	3 86	0 285	0 496	2 21	0 240	0 540	6 14	0 349	0 434
Average.	4 24	0.263	0.448	2 94	0.231	0 468	5 95	0 307	0 421

* Values expressed in milliequivalents of N per liter

siderable; in ten males the α-amino N in plasma ranged from 3.15 to 4.70 mg. per cent (average 4.38) and in nine females the values varied from 2.95 to 3.99 (average 3.43). In whole blood the range for males was 4.19 to 7.81, for females 4.34 to 6.35 mg. per cent. α-Amino nitrogen in red blood cells varied from 6.85 to 11.16 in men and 6.29 to 10.79 mg. per cent in women. For the whole group the standard deviation was 0.66 for plasma, 0.94 for whole blood, and 1.58 for erythrocytes.

The free α-amino acid N of plasma observed by us is perhaps slightly lower than that reported by others (1, 5). More discordant is the fact that we find a difference in values between the sexes, although it does not appear to be statistically significant.

Glycine constituted 6.2, 8.0, and 5.2 per cent of the free α-amino N in whole blood, plasma, and red cells respectively (Table II). The figures

for alanine were 10.6, 16.1, and 7.1 per cent respectively. Together they comprise a relatively large fraction (24 per cent) of the total free α-amino acid nitrogen in human plasma. This finding is not in accord with the recent reports of Hamilton (2) and Hier and Bergeim (3), whose data, taken together, leave little or no room for alanine and glycine or for aspartic acid, cystine and cysteine, methionine, proline, and serine, which have not been determined. According to the former authors, glutamine constitutes from 18 to 25 per cent of the free α-amino acid N of plasma. The latter observers account for 76 per cent of the total amino N in the form of ten other "essential" and "non-essential" amino acids. It is difficult to ascribe this discordance to erroneously high values reported for glutamine. It is more likely referable to the microbiological assay methods employed by Hier and Bergeim (3), by which no distinction between free and bound amino acids is shown, whether the latter are "conjugated" or in peptide combinations (8, 11).

Glycine and alanine comprise a smaller percentage of the free α-amino N of erythrocytes, despite the fact that the concentration of glycine is greater, and of alanine no less, in the cells than in the plasma.

The free amino N of red blood cells is about twice as great as that of plasma. This is in accord with the observations of other observers, who, using the same technique (1, 8, 12), report values which range from 6.5 to 9.6, averaging about 7.7 mg. per cent. As has been pointed out by other observers (7, 8) glutathione contributes largely to the high amino N values obtained for erythrocytes.[3] It seems unlikely, however, that this is the whole explanation of the discrepancy between erythrocyte and plasma amino N, since, at most, red cell glutathione amino N (as measured by the determination of "bound" glycine (8) or the analysis of the glutathione directly (13, 14)) comprises only 2 to 3 mg.; if this is deducted from the total free α-amino N values for red cells, we are still left with a concentration of free α-amino non-glutathione N exceeding that in plasma. It should be noted, also, that irregularities in the recovery of glutathione infiltrates of biological material deproteinized by agents other than sulfosalicylic acid (8, 13) would tend to give erroneously low values for the α-amino N of erythrocytes which have been rendered protein-free by tungstic or picric acid. Accordingly, the difference between the free, non-glutathione, α-amino N of erythrocytes and plasma is probably even greater than indicated above.

If from our observed values for red cell amino N (6.06 milliequivalents) we deduct the value of red cell glutathione obtained by others (2.14 milliequivalents of α-amino N), we are left with 3.92 milliequivalents of non-glutathione α-amino N, of which glycine constitutes 7.8 per cent and al-

[3] The concentration of glutathione in plasma is negligible (8, 13).

anine 10.7 per cent.   The ratio of glycine to non-glutathione α-amino N in the erythrocyte thus is practically the same as that in plasma (8.0 per cent), whereas for alanine the relative concentration is greater in the plasma (16 per cent) than in the cells (10.7 per cent).   As far as we have been able to ascertain, similar studies have been made for only one other amino acid,

TABLE III

Blood Glycine and Alanine in Non-Fasting Normal Subjects

Analytical values expressed in mg. per cent.

Subject	Hrs. post cibum	Glycine			$\frac{C_g}{P_g}$	Alanine			$\frac{C_a}{P_a}$	Hemato-crit cells
		Blood	Plasma	Cells		Blood	Plasma	Cells		
										per cent
G. L.	0	2.00	1 64	2.50	1 52	2.74	1.75	4.09	2.34	42.0
	2.00	2.30								
	2.00	2.36								
	2 00	2 72	1.93	3.81	1.97	4 50	4 00	5 20	1.30	42.0
M. M.	1.50	3.06	1 96	4 28	2 18	3 32	2.15	4 86	2.26	43.0
	0	1.95	1.77	2.22	1.26	4 00	4.20	3.71	0.88	41.0
B. A.	0	1 98	1.75	2.20	1.25	2.16				50.0
	1.25	2 42	1.96	2 88	1 47	4.16	4 40	3.92	0 89	50.0
	0*	2 42	2.32	2.52	1.09	3 00	2.50	3.50	1.40	50.0
	0.50	2.60	2.00	3.20	1.60	2 34	2.68	2 00	0 75	50.0
	1.00	2 86	2 24	3.48	1.55	4.18	4 32	4 04	0.94	50.0
	2.25	2.80	2 18	3.42	1 57	3 84	3.84	3 84	1 00	50.0
	3.25	2 64	2.06	3.22	1.56		4.58			50 0
J. L.	2.00	1.97				3 42				
F. R.	2.00	2 26	1 72	3.13	1 82	3 84	4 16	3 31	0.80	38 0
E. L.	0 50	1.86	1.55	2 18	1.41	3 34	3.07	3.60	1 17	49.5
W. R.	1.00	1.86	1 56	2.24	1.43					44 0
G. G.	0.55	2.35	2.02	2 74	1 36	6 40	4.16	9 00	2 15	46 0
I. E.	1 30	2.10				4.41				
S. C.	1 25					3 49	3 71	3 21	0 87	43.0
Average of values during absorptive state . .		2 41	1.93	3 12	1.63	3 94	3 73	4 30	1 15	

* This blood was obtained immediately before dinner, 3 hours after a light breakfast.   The time relationships of the subsequent analyses are based on this determination.

tryptophane, whose concentration in cells is only one-fifth of that in plasma (12).   This evidence suggests that the pattern of amino acids with respect to their relative concentrations is different in the plasma from that in the erythrocyte.

Blood glycine concentrations were higher in non-fasting than in fasting subjects (Table III).   This was observed particularly in the cells, resulting

in an increase in the concentration ratio of cell to plasma glycine. On the other hand, no significant difference was observed between the alanine concentrations of fasting and non-fasting blood.

Following the ingestion of glycine its concentration rises rapidly in plasma and erythrocytes, attaining its highest peak in from 1 to 1½ hours (Fig. 1).
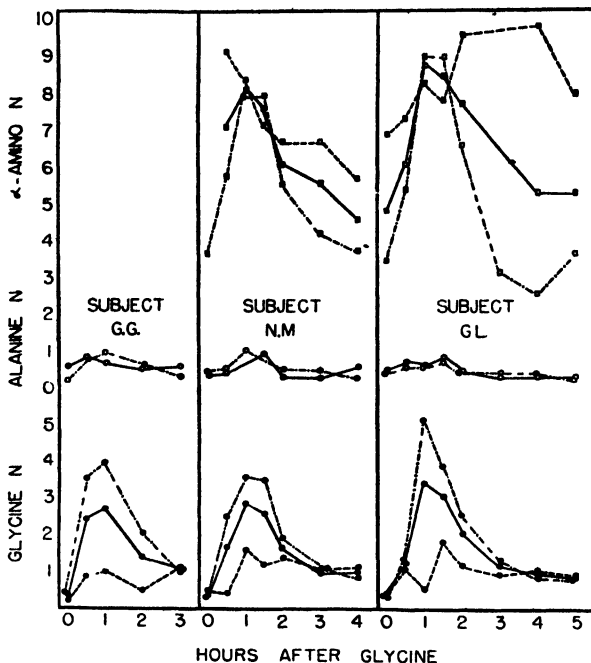
FIG. 1. Glycine N, alanine N, and total free α-amino N following ingestion of glycine. ● represents glycine N; ○ alanine N; □ α-amino N. All values are given in mg. per 100 cc. The solid lines indicate whole blood; the broken and dotted lines, plasma; the dash lines red blood cells. As will be noted in the experiment on N. M., the control value of the α-amino N for whole blood is unfortunately missing, due to a technical error encountered in that analysis. In view of evidence (17) indicating that blood α-amino N returns essentially to normal within 3 hours following the oral or parenteral administration of amino acid mixtures, there might be some justification for considering the 4 hour value of the above subject in lieu of the control.

That glycine is easily absorbed from the gastrointestinal tract is thus indicated. This is in accord with the early observations of Folin and Berglund (15), who reported a rise of 3 mg. of amino N within 2 hours in the blood of a subject who had received 1.2 gm. of glycine per 10 pounds of body weight. Thereafter, the level declines rapidly (Fig. 1) but does not return to the preingestion value within 5 hours. The early rise in glycine is greater in the plasma than in the red blood cells, resulting in a marked

decrease in the concentration ratio of cell to plasma glycine.   This suggests either that glycine does not enter the erythrocyte readily or that the amino acid is rapidly converted there to something else.   That red cell amino N rises much more than red cell glycine after the ingestion of this amino acid (Fig. 1) supports the latter view, if we consider that glycine may be rapidly converted in the cells to glutathione.

The speed with which ingested glycine is absorbed into the blood stream has been referred to above.   But the slowness with which the blood is completely cleared of its added glycine is noteworthy and is in discord with the behavior of most amino acids, which are rapidly removed (16, 17). Other observers have similarly noted the slowness with which the blood amino N returns to its control level following the ingestion of glycine. The administration of this amino acid apparently results in its accumulation in other tissues also.   It has been shown that of most amino acids only glycine causes a rise in the amino N of muscle; in liver, it caused a greater rise in amino N than any other amino acid (18).   These facts are also consistent with evidence (19, 20) that glycine, in contradistinction to other amino acids, is deaminated and oxidized with great difficulty by tissue slices or suspensions of broken cells.[4]

On the other hand, it has been conclusively demonstrated by means of glycine tagged with carbon isotopes that its metabolism starts immediately following its administration, since isotopic carbon dioxide soon appears in the expired air (22).   Nevertheless, glycogen formation from glycine does not take place till 6 hours after its administration (23).

It is conceivable that the slowness with which ingested glycine is completely cleared from the blood is due in part to delayed excretion into the urine.   Our observations indicate that only very small amounts of glycine are excreted, even when there are large amounts in the blood.   After the administration of this amino acid to two subjects (G. G., N. M.) only 38 and 43 mg. were recovered in the urine in $3\frac{1}{2}$ and 5 hours respectively. One of these individuals excreted 98 mg., the other 148 mg. during 24 hours on another day, when the intake was derived solely from the diet (Table IV).   It is unlikely, therefore, that the ingestion of glycine would greatly augment this figure.   The per cent of the dose which appears in the urine (less than 1 per cent) is less than the values reported for the excretion of other amino acids.   In dogs, 16 per cent of the amino N of a casein hydrolysate is excreted within 1 hour (16), and, when pure amino acid mixtures are given orally and intravenously, 3 to 5 per cent and 2 to 16 per cent, respectively, are excreted in the urine (17).   The reason for the small

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[4] Although Ratner et al. (21) have recently described a specific enzyme, occurring in many animal tissues, which converts glycine to glyoxylic acid by oxidative deamination, there is no evidence that this occurs in intact tissues.

excretion of glycine following its ingestion may well be a reduction in glomerular filtration consequent to elevation of the plasma glycine concentration (24, 25). It should also be noted that of the four amino acids, glycine, alanine, glutamic acid, and arginine, the rate of renal tubular reabsorption is highest for glycine (25), which also may account for the relatively small excretion of this amino acid in urine.

The average amount of glycine excreted in the urine in 24 hours is 120 mg. (Table IV). The variation from individual to individual seemed independent of the sex, the volume of urine passed, its specific gravity, and the composition of the diet (carbohydrate, protein, or fat). One subject (A. R.), however, who excreted 227 mg. of glycine, had ingested the largest number of calories of our series; a large fraction of the caloric intake came from protein and fat.

TABLE IV

Glycine Excretion in Normal 24 Hour Urine

Subject	Urine volume	Glycine	Total glycine	Calories ingested
	cc.	*γ per cc*	*mg.*	
G G., ♂	1049	93.0	97 7	
	1135	72 8	82 6	2223
N. M., ♀	2350	63 8	148 0	1656
W B , ♂	1065	115 0	122 5	1953
	945	109 2	103 1	1432
I E , ♀	930	117 4	109.2	1635
	1330	93 8	124.8	1530
L. R., ♀	755	127 2	96 2	1643
G. L , ♀	1173	75.0	88 0	
A. R , ♂	1158	196 0	227 0	3011
Average			119 9	

Erythrocyte and plasma alanine also rise slightly following the administration of glycine (Fig. 1); although the change is small, it is significantly outside the limits of error of the analytical method. The explanation of this observation is obscure.

In two cases studied (N. M. and G. L.) the free α-amino N of the blood paralleled the changes in glycine and alanine. 1 hour after glycine was taken, when changes in glycine concentration were maximal, the rise in α-amino N could be accounted for almost completely by the rise in glycine plus alanine. At this point, the difference between the rise in amino N and the increase in glycine plus alanine was 0.4 to 0.5 mg. of N. Occasionally thereafter discrepancies of as much as 1.83 mg. of N were observed.

We are indebted to Miss Norma Miller for technical assistance.

SUMMARY

1. The concentrations of glycine, alanine, and free α-amino acid nitrogen in normal blood, plasma, and red blood cells have been established.

2. Glycine plus alanine constitutes 16.8, 24.1, and 12.3 per cent of the amino N in whole blood, plasma, and erythrocytes respectively.

3. Following the ingestion of glycine, the concentration of this amino acid in the blood increases rapidly, then declines, but returns to its previous level slowly. There is a small concomitant rise in blood alanine. The changes in free α-amino nitrogen parallel those of glycine plus alanine.

4. The urinary excretion of glycine during 24 hours, and after its ingestion, is small.

5. The possible significance of these observations is discussed.

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A MICROCOLORIMETRIC METHOD FOR THE DETERMINATION OF MANGANESE IN BIOLOGICAL MATERIALS WITH 4,4'-TETRAMETHYLDIAMINOTRIPHENYLMETHANE

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In studies of manganese deficiency in the rabbit,[1] a method for the determination of this element in small samples of blood serum and other tissues was needed. The most promising of the organic reagents suitable for manganese appeared to be 4,4'-tetramethyldiaminodiphenylmethane, which has been used by Harry (1) in a colorimetric procedure and by Mellan (2) as a spot test reagent. Preliminary tests showed that 4,4'-tetramethyldiaminotriphenylmethane is approximately 10 times as sensitive as the diphenyl compound; consequently this investigation was limited to the triphenyl compound.

The method consists of a periodate oxidation of manganese to permanganate with subsequent development of a yellow color by the reaction of permanganate and 4,4'-tetramethyldiaminotriphenylmethane.

Factors influencing the reactions involved were found to be the kind and concentration of acid used, the concentration of periodate, and the temperature and time of reaction between the reagent and permanganate. An acid mixture of 0.6 N in HNO_3 and 3.2 N in H_3PO_4 was found to be satisfactory. The concentration of periodate is kept sufficiently constant by measuring the required amount of sodium periodate with a bowl-shaped glass scoop.

Oxidation of the reagent by periodate is appreciable at a temperature of 90–100° and is slight by permanganate at temperatures of 20–40°. If the reagent is added to the permanganate-periodate solution at 75–85°, the reaction with the periodate is not appreciable, while the reaction with permanganate is sufficient to yield a good color. A temperature of 80° was therefore chosen as most suitable.

The measurement of the final color is made by means of a photoelectric colorimeter of the Evelyn type (3) with matched test-tubes 18 mm. in diameter. The course of the reaction is followed by noting the galvanometer reading, and the lowest per cent transmittance is taken as the final reading.

[1] Ellis, G. H., Smith, S. E., and Gates, E. M., unpublished work. Smith, S. E., and Ellis, G. H., unpublished work.

Method

Reagents—

Water. Redistil water from an all-Pyrex still.

Nitric acid. Redistil a 1:1 mixture of c.p. concentrated HNO_3 and water from an all-Pyrex still.

Phosphoric acid. c.p., 85 per cent. Different bottles of the same lot as well as different lots of phosphoric acid from the same company have been found to vary considerably in their manganese content. Choose a supply giving a low blank.

Acid mixture. To 600 ml. of 1 N HNO_3 (redistilled), add 75 ml. of 85 per cent H_3PO_4 and make to 1 liter with redistilled water.

Sodium periodate. The grade supplied by the Eastman Kodak Company is satisfactory.

Color reagent. Make a 0.1 per cent solution of 4,4'-tetramethyldiamino-triphenylmethane in 5 per cent, by weight, H_3PO_4. The reagent, as obtained from Eastman, can be used without further purification. This solution should be prepared daily and must be absolutely colorless.

Standard manganese solution. Dissolve 0.144 gm. of c.p. potassium permanganate in about 100 ml. of redistilled water and reduce with sulfur dioxide, as suggested by Richards (4). Boil off the excess sulfur dioxide and, after cooling, dilute to 1 liter. 1 ml. of this solution contains 0.05 mg. of manganese, and a suitable working standard is prepared weekly from this stock solution.

Preparation of Samples. Bones—Ash in a covered porcelain or silica dish for 18 hours at 600°. Add sufficient 1:1 HNO_3 (redistilled) to dissolve the ash and evaporate to dryness on a hot-plate. Reash at 600° for another 18 hours. Repeat if necessary to get a white ash.

Blood or Blood Serum—Dry the sample in a porcelain or silica dish in a drying oven or on a steam plate. Start with a cool furnace, raise the temperature gradually to 600°, and hold for 18 hours. Add 3 ml. of 1:1 HNO_3, take to dryness, and reash at 600° for a few hours.

Liver—It is usually difficult to ash liver, and a few ml. of 20 per cent magnesium nitrate added to the sample prior to ashing are helpful. Ash at 600°, as above. It is sometimes necessary to resort to three HNO_3 treatments before a white ash is obtained.

Procedure

Dissolve the ash in the least possible volume of the nitric-phosphoric acid mixture, warming gently to hasten solution. Transfer an aliquot of this solution containing 0.05 to 0.5 γ of manganese to a 20 ml. glass-stoppered test-tube, make to 10 ml. volume with the acid mixture, and add 50 mg. of $NaIO_4$ (a glass measuring scoop is sufficiently accurate). Heat for 1.5 hours in a boiling water bath. Transfer two 3 ml. aliquots to color-

imeter tubes, cover with an inverted micro beaker, and place in a water bath held at 80 ± 1.0° for at least 10 minutes. Remove a tube from the bath, immediately add 3 drops of the 4,4'-tetramethyldiaminotriphenyl-methane solution, shake gently for 5 seconds, place in the colorimeter, and note the minimum galvanometer reading. The filter used is made up of Corning standard thickness Filters 3387 and 3962 and half standard thickness Filter 5113. A complete set of standards must be run simultaneously. Reagent blanks should read 90 per cent transmittance or higher.

DISCUSSION

The determination is most conveniently carried out by two persons, one to operate the colorimeter and record the results and the other to prepare the samples for the colorimeter.

Contamination is a big factor in reproducibility; samples should be covered at all times to be protected from dust. During ashing Pyrex watch-glasses may be used. All glassware should be cleaned just prior to use with hot concentrated HNO_3 and rinsed with distilled water, followed by a small amount of redistilled water. Before the initial reading and between subsequent readings the colorimeter tubes should be cleaned by rinsing with distilled water, followed by hot acid-mixture to which $NaIO_4$ has been added. After draining, the tubes are ready for use.

If samples are too concentrated, it is unnecessary to repeat the entire periodate oxidation if the sample is diluted with acid-mixture which has been boiled with $NaIO_4$. The diluted sample is then heated for 10 minutes in the boiling water bath before aliquoting.

Upon addition of the reagent, the rate of development and of fading of the yellow color depends upon temperature, light intensity, and concentration of manganese. When the reaction is carried out according to the procedure described above, maximum color of the blanks develops in about 3 minutes, upon addition of the reagent, and then remains constant for about 5 more minutes. Standards containing 0.5 γ of Mn per 10 ml. reach a maximum color in about 30 seconds and remain constant for about 45 seconds more. The rate of color development and fading is the same with standard manganese solutions or with samples of bone, liver, Canada field pea hay, blood serum, and milk, indicating that interfering substances are absent from these materials.

Since the color reaction is influenced by strong light, this work was carried out in a darkened room with the light covered by yellow cellophane.[2] After fading for a half-hour or so, the yellow color remaining is stable for at least 20 hours. This stable color, however, did not prove as satisfactory for quantitative work as the maximum color.

[2] No. 300 PRC tango, du Pont.

Various organic solvents listed in Table I will extract the yellow color and yield a blue solution in the non-aqueous phase, or, in the case of some

TABLE I

Color Reactions with Organic Solvents

An equal volume of solvent added to a deep yellow-brown solution produced by the reaction of permanganate and 4,4'-tetramethyldiaminotriphenylmethane.

Solvent	Reaction	
Single phase		
Ethanol	Colorless	
Acetone	No change	
1,4-Dioxane	Clear blue	
Pyridine	" "	
Isopropyl alcohol	" "	
Double phase	Aqueous phase	Solvent phase
Skellysolve	Yellow	Colorless
Xylene	"	"
Carbon tetrachloride	"	"
Diethyl ether	"	"
Isoamyl acetate	"	"
Cyclohexane	"	"
Benzene	Deep yellow	Faint blue
Chloroform	Faint "	Blue
Isobutyl alcohol	Colorless	"
Cyclohexanol	Yellow	"
Toluene	Deep yellow	Faint blue
Ethylene dichloride	Colorless	Blue

TABLE II

Comparison of Manganese Values on Various Materials by Periodate and 4,4'-Tetramethyldiaminotriphenylmethane Methods

Sample	Periodate method	4,4'-Tetramethyldiaminotriphenyl-methane method
	γ per gm.	*γ per gm.*
Soy bean hay	69.0	70.0
	57.5	59.8
	55.5	56.4
Liver	0.375	0.385
Bones	3.13	3.43
	2.77	3.35
	16.4	16.4
	7.88	8.72
	11.8	12.8
	14.4	14.4
	6.71	6.71
	11.6	10.6

water-miscible solvents such as isopropyl alcohol and 1,4-dioxane, a blue homogeneous solution is produced. The intensity of the blue color is

TABLE III

Recoveries of Added Manganese

Sample	10 ml. solution	Added	Found	Recovery
	γ	γ	γ	*per cent*
Liver	0.094	0.050	0.142	96 0
			0.145	102 0
			0 150	112 0
Soy bean hay	0.215	0.200	0.405	95 0
		0.050	0.260	90 0
Bone	0.170	0.200	0.350	90 0

TABLE IV

Comparison of Manganese Values on Bone Samples Analyzed on Different Dates and with Different Aliquots

Bone No.	Apr 15, 1946		Apr 18, 1946	
	Aliquot analyzed	Mn per 10 ml.	Aliquot analyzed	Mn per 10 ml
	ml.	γ	*ml.*	γ
1	5	0.134	10	0.137
6	3	2.160	1	2.630
2	5	0.100	10	0 110
7	3	1.720	1	1.670
9	5	0.116	10	0.115
12	3	0 790	3	0.783
16	5	0 118	10	0.109
19	3	1 425	3	1.265
20	5	0.168	10	0.169
23	3	1.465	3	1.483
35	5	0 160	10	0.155
37	3	Over 2 7	1	3 560
45	5	0.118	10	0.128
48	3	1.400	3	1.435
47	5	0 150	10	0.148
49	3	2.310	1	2 810
50	5	0 062	10	0.086
51	3	1.345	3	1.355
53	5	0.088	10	0.086
54	3	1 000	3	0.980

roughly proportional to that of the yellow color and is somewhat more intense. If the need should arise for a more sensitive method, the use of such organic solvents offers a possible solution.

Results

The validity of the method was tested by a comparison with the periodate
method (5), as shown in Table II.   Since results by the two methods were
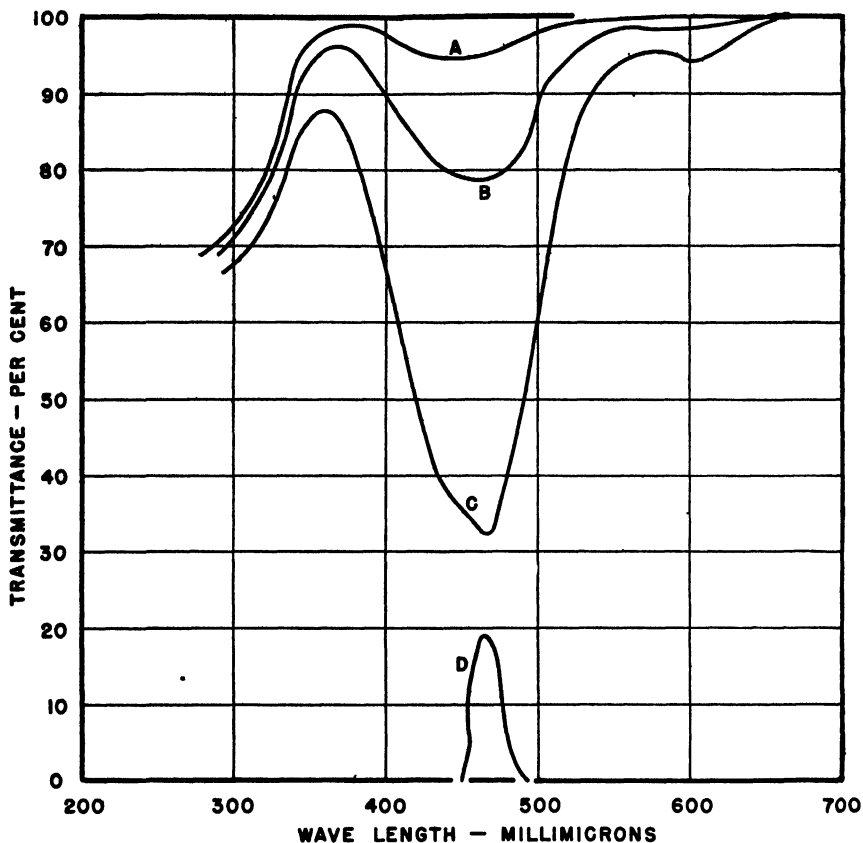
Fig. 1. Spectral transmittance curves· for 4,4'-tetramethyldiaminotriphenyl-
methane with manganese and the filter combination used in the determination of
manganese.   Curves were determined on the stable color with the Beckman spectro-
photometer with 1 cm. cells.   Curve A, reagent blank; Curve B, 0.2γ of manganese
per 10 ml.; Curve C, 0.5 γ of manganese per 10 ml ; and Curve D, light transmitted
by combination of Corning filter Nos. 5113 (0.5 standard thickness), 3387, and 3962
(standard thickness).

in good agreement, no extensive tests for interfering substances were made.
However, negative results were obtained with the following substances:
iron, lithium oxalate, lead acetate and nitrate, copper acetate and sulfate,
ammonium hydroxide and sulfate, magnesium sulfate, and silver nitrate.

The following substances gave a yellow color: chromium trioxide, ammonium vanadate, ceric ammonium sulfate, and ceric sulfate. The chloride ion also interferes and all samples containing chloride must be evaporated to dryness with 1:1 HNO_3. Cerium and vanadium are not likely to be present in biological material to any appreciable extent, but care must be taken not to use glassware cleaned in chromic-sulfuric acid cleaning solution.
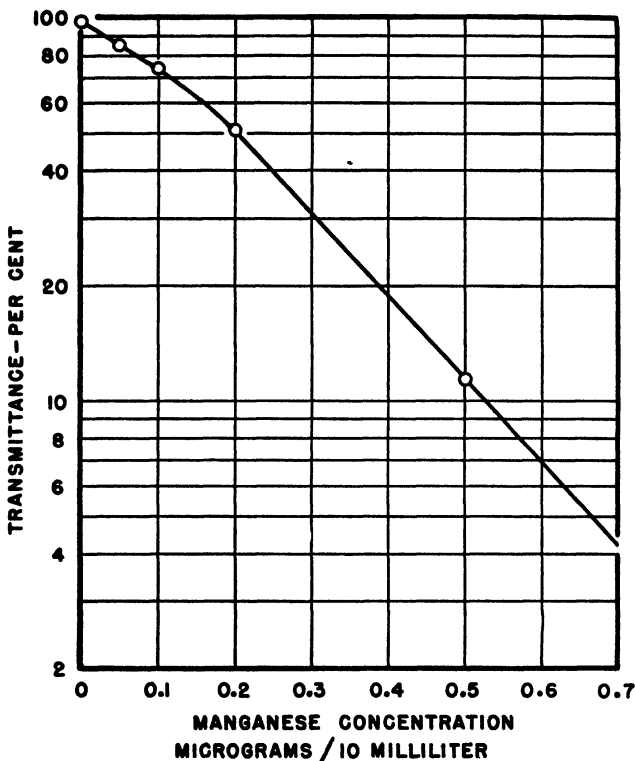
FIG. 2. Typical manganese standard curve

Recoveries of manganese added to various materials ranged from 90 to 112 per cent (Table III). The data of Table IV show the reproducibility of the method carried out on different days with different sized aliquots of bone samples.

The manganese method of Wiese and Johnson (6) was found by the authors to give erratic results when applied to samples containing less than a few micrograms of manganese. This is believed to be due in part to the difficulty found in obtaining samples of sodium bismuthate giving

a sufficiently low blank and in part due to contamination of the sample during filtration. Of six lots of sodium bismuthate obtained from various sources, only one gave a reasonably low blank, but even in this case the blank was considerably greater than that obtainable in the present procedure.

The absorption curve for the yellow color, as determined with a Beckman spectrophotometer, is shown in Fig. 1. The transmission of light by the colorimeter filter combination is also shown in Fig. 1.

The standard curve in Fig. 2 shows that Beer's law is not followed.

SUMMARY

A microcolorimetric method for the determination of manganese in biological materials with 4,4'-tetramethyldiaminotriphenylmethane is presented. Results obtained by this method are comparable with those obtained by the periodate method, and satisfactory recoveries of added manganese are shown. The method is sensitive from 0.02 to 0.5 γ of Mn per 10 ml. of solution, and results are reproducible on different days.

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THE UPTAKE OF RADIOACTIVE PHOSPHORUS BY THE CALCIFIED TISSUES OF NORMAL AND CHOLINE-DEFICIENT RATS*

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In this laboratory *in vivo* studies of the uptake of phosphate containing radioactive P^{32} by bone suggested that there is an appreciable ionic exchange between bone and the circulating fluids (1). In a new series of experiments designed to observe the effect of dietary choline on bone metabolism, radioactive phosphorus, P^{32}, was again employed. The newly acquired data have led to a reexamination of the magnitude of the ionic exchange process and have permitted a more accurate description of the mechanism by which radioactive phosphorus is transferred from the plasma to bone.

EXPERIMENTAL

Two groups of fifty male and two groups of fifty female rats were weaned and placed on an adequate synthetic diet (2). Young rats were employed because they are particularly susceptible to choline deficiency (3), and thus represented the type of experimental animal most likely to show bone changes.

After 24 hours on the control diet to accustom all of the animals to a synthetic provision, one group of fifty males and one group of fifty females were placed on a choline-deficient diet. The choline-deficient was the same as the normal diet, except for the exclusion of the choline supplement and the inclusion of 0.5 per cent guanidoacetic acid.

After 3 days on the synthetic provisions all animals were injected intraperitoneally with inorganic phosphate containing P^{32} and sacrificed in groups of five at intervals ranging from $\frac{1}{2}$ to 16 hours after the injection.

The femurs were removed and dissected free of flesh; the diaphyses were grossly separated from the epiphyses and the metaphyses (hereinafter designated femur ends), and the material from each group of five rats was pooled. The pooled samples, after consecutive 24 hour extractions with

ethyl alcohol and ether, were ashed by boiling in 3 per cent potassium hydroxide in ethylene glycol to dissolve the organic matrix (4). Each sample of bone ash was dried to constant weight at 120° after washing with boiling water. The dried samples were dissolved in 2 N hydrochloric acid and diluted to suitable volumes for phosphorus (5) and radioactivity analyses. Radioactivity measurements were made on Bale immersion type, scale-of-four, Geiger-Müller counters (6). The acid-soluble inorganic phosphorus from pooled blood samples of each group of five rats was isolated as the strychnine molybdate (7) for analysis and radioactivity measurement.

The P^{32} measurements were expressed as "specific activity coefficients" which are equal to the counts per minute per gm. of phosphorus divided by the counts per minute administered per gm. of body weight.

Results

In vitro studies in this laboratory[1] have indicated conclusively that the inorganic substance of bone is capable of exchanging phosphate when suspended in a medium containing dissolved phosphate. With the *available* experimental methods, it is impractical to avoid such contamination of the inorganic phase of bone with high activity phosphate present in the tissue fluids which bathe the whole bone. It was necessary, therefore, to investigate the magnitude of P^{32} exchange which occurs during the ashing process.

The femurs of two rats were freed of adhering matter, covered with 25 ml. of 3 per cent potassium hydroxide in ethylene glycol, and allowed to simmer for 90 minutes. Then, 0.5 ml. of an aqueous solution containing radioactive phosphate (10,000 counts per minute = 2 γ of phosphorus) was added and the solution allowed to simmer for another 90 minutes. The supernatant was decanted and the ashed bone washed repeatedly with small portions of distilled water. The washings were added to the glycol solution and made to a convenient volume. The ashed bone was dissolved in approximately 2 ml. of 3 N hydrochloric acid and made to volume. Both the glycol and the solutions of bone ash were analyzed for phosphorus and radioactivity. The results are presented in Table I. From the data reported in Table I, it can be seen that when the initial radioactivity of the ash = 0 (just at the time of addition of the tracer) and the radioactivity of the phosphate in the glycol = 10,000 counts per minute (just at the time of addition of the tracer), further glycol treatment transfers on the average 25 per cent of the activity to the ash phase.

If it is assumed that the phosphate extracted by glycol has the same specific activity coefficient as that of inorganic phosphate of blood (a maximum value), a correction can be applied to results from *in vivo* experiments to

reduce the error introduced by the transfer of activity during glycol extraction by means of the following equation:

$$\frac{\text{Counts per minute in bone ash} -0.25 \text{ (specific activity of blood inorganic P} \times \text{mg. P in glycol)}}{\text{Mg. P in bone ash}} = \begin{array}{l}\text{corrected specific activity} \\ \text{of bone ash}\end{array}$$

Uptake of P³² by Femur of Young Normal Rats—The radioactivity measurements obtained from the study of the inorganic phosphate of blood and of the two portions of femur are presented in Fig. 1. Both corrected and uncorrected data are given. During the first few hours the specific activity coefficient of the circulating inorganic phosphate fell very rapidly. By 16 hours, the specific activity coefficient was decreasing quite slowly. On the other hand, activity appeared quickly in the phosphate of both portions of

TABLE I

Distribution of Radioactive Phosphate between Bone Ash and Glycol-Soluble Material

Femur ash weight	Femur ash P	Glycol P	Total P³² added in bone ash
mg	*mg.*	*mg.*	*per cent*
36.4	5 87	0 62	34.9
35.7	5 79	0.56	24 5
25 7	4.08	0 61	16 0
28 0	4 41	0 56	24 8
Average			25 0

bone, but the level remained high and relatively constant in spite of the concurrent rapid fall in the activity of the inorganic phosphate of blood.

The important difference between the corrected and uncorrected data which are presented in Fig. 1 is the slower rise in the corrected activity of the phosphate of bone ash in the early time periods.

Uptake of P³² by Femur of Young Choline-Deficient Rats—To provide comparisons between control and deficient animals with respect to both sex and uptake of radioactive phosphorus, the data obtained on choline-deficient rats are plotted in Fig. 2. All specific activity coefficients have been corrected for error contributed by the extraction with ethylene glycol (*vide supra*). It is evident from these data that there are no consistent effects of mild choline deficiency on the uptake of radioactive phosphate by bone of either sex.

In an earlier experiment, the results of which have been reported elsewhere (8), another group of rats in an extreme state of choline deficiency was examined for differences of uptake of radioactive phosphorus. Both

the tibias and femurs of the choline-deficient animals showed only slightly but consistently lower isotope levels than those of the controls. At the same time the isotope levels of the circulating *blood* of the deficient animals were somewhat higher than the controls. We now consider that the ad-
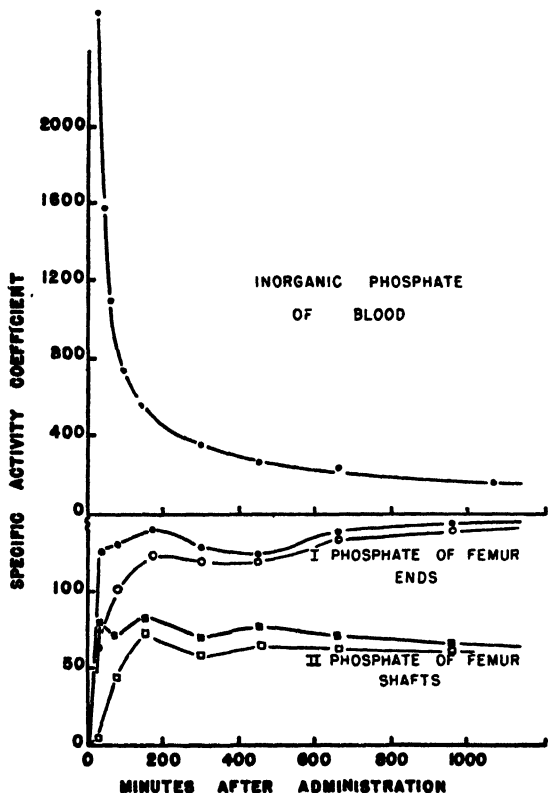
FIG. 1. Graphs showing the relationships between the concentrations of labeled phosphate in blood, femur ends, and femur shafts and time after administration of the isotope. The activity of labeled phosphate is expressed as the specific activity coefficient which is equal to counts per minute per gm. of phosphorus divided by counts per minute administered per gm. of body weight. The solid circles and solid squares for femur ends and shafts are *observed* values; the open circles and open squares are calculated values corrected for exchange during glycol ashing

vanced kidney dysfunction and the moribund state of the experimental animals invalidated the results.

State of Calcification of Choline-Deficient Rats—Since the radioactivity results are inconclusive, corollary data have been examined to evaluate the effect of choline deficiency on bone metabolism. The body weight changes

and the weights of the inorganic ash of the femur and tibia of rats made more severely deficient in the earlier experiment are presented in Table II.
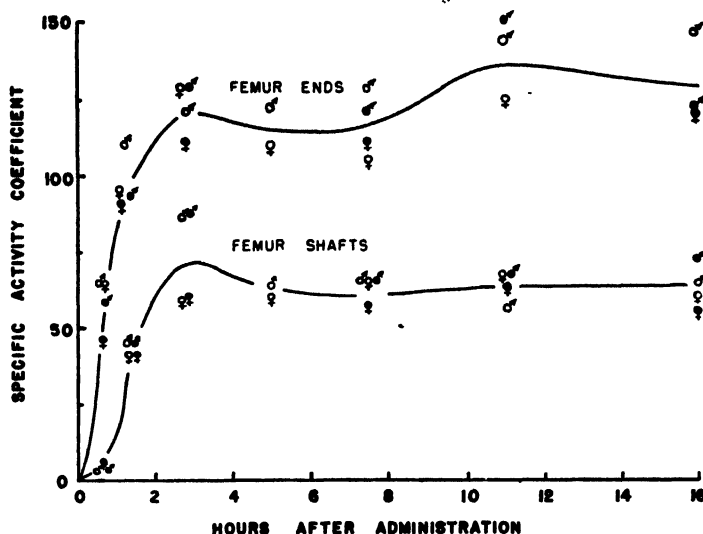
FIG. 2. Data showing the lack of an effect of mild choline deficiency on the uptake of labeled phosphate by femurs of male and female rats. ♂ and ♀ denote normal animals; the solid symbols, deficient animals. All data are calculated values corrected for exchange during glycol ashing.

TABLE II

Body Weights and Weights of Inorganic Ash of Femurs and Tibias of Normal and 5 Day Choline-Deficient Rats

Group	No of animals	Body weight			Femur		Tibia	
		Initial	Final	Gain per 5 days	Ash weight	Ash weight	Ash weight	Ash weight
		gm.	*gm.*	*gm.*	*gm.*	*per cent of body weight*	*gm.*	*per cent of body weight*
Control	16	33 8	43 4	9.6	0 0282	0.634	0.0263	0.615
S.D.*				2.2		0.072		0.040
Deficient	15	34.2	39.3	5.0	0 0284	0.738	0.0297	0.757
S.D.*				2 5		0 046		0.053

* Standard deviation of the mean.

These data illustrate the comparative states of calcification in the normal and 5 day-deficient rat.

The average weights of the femur and tibia of the deficient animals were equal to or slightly greater than those of the controls. This indicates that

the femur and tibia have grown at a normal rate in spite of the general inanition of the deficient animals. Thus, we have obtained no direct evidence of an effect of acute choline deficiency on the calcification of bone in the rat.

DISCUSSION

These data on the uptake of P^{32} by bone are qualitatively similar to those of Manly *et al.* (1) and Cohn and Greenberg (9). The previous work indicated that a rapid equilibration occurs between the phosphate of blood and that of bone. However, the method of administration (oral) and the time periods chosen for study (the earliest being 2 hours) prevented these workers from recognizing the extreme rapidity with which this equilibration takes place.

Manly *et al.* (1), in picturing the normal events which take place in bone as interpreted from their data, were led to assume, "That calcified tissues are composed physiologically of two portions, one of which rapidly comes to equilibrium with certain blood minerals . . . The other portion reaches equilibrium slowly and, by comparison, exhibits negligible exchange with these blood minerals." The rapidly equilibrating fraction was designated as "labile" bone, the fraction with negligible exchange as "stable" bone. Also of some importance to the present discussion is their observation that "the relative amounts of labile to stable calcification differ widely in epiphyses, diaphyses, incisors, and molars." Further consideration leads us to question the validity of the assumptions of Manly *et al.* relating to labile and stable bone, and to propose a modified picture more in keeping with the available data.

While it is perfectly conceivable that phosphate located in the surface of the individual crystals of apatite in the bone can undergo exchange with phosphate in the surrounding fluid, it would seem thermodynamically impossible that phosphate in the center of the crystals could exchange in a finite time without complete dissolution of the crystals themselves.[2] From data in the literature (10) on the estimated dimensions of the individual apatite crystal it can be calculated that total phosphate of the order of one-fifth or 20 per cent is located in the surface.

It is hardly possible that the rapid accumulation of tracer in the bone is due to normal growth processes. The amount of radioactive phosphorus which would appear in the femur ends as a result of accretion would account

[2] Primitive Haversian systems are known to undergo degeneration normally at some places, and, following degeneration, to give second generation Haversian systems. This process involves the dissolution of apatite crystals and reordering of the organic matrix. The magnitude and rate of this combined alteration are comparable to accretive processes rather than ionic exchange and decrease throughout the life of the animal.

for only about 8 per cent[3] of the observed activity at 16 hours. Furthermore, it has been shown (11) that the specific activity of the phosphorus of a humerus whose growth has been hindered by unilateral section of the brachial nerve is identical with that of the normally growing, contralateral humerus in the same animal. This situation obtains in spite of an observable decrement in the rate of accretion of bone salt in the denervated limb as compared with that of the normal limb.

Perhaps the simplest explanation of the rapid, initial uptake of tracer following intraperitoneal administration is that an exchange-adsorption[4] occurs between the inorganic phosphate of the plasma and the phosphate ions in the surface of the bone salt adjacent to the circulating fluid. Such an exchange-adsorption reaches a steady state very rapidly.

Paneth and Vorwerk (12) obtained a nearly steady equilibrium in less than 1 minute when finely divided lead sulfate was shaken with a saturated solution of the same salt containing the lead isotope, thorium B. Since the experiments reported here were completed, *in vitro* studies in this laboratory[1] have indicated conclusively that there is a rapid exchange of radioactive phosphate of a buffer solution and the phosphate of ashed bone powder suspended in it. Calculations based on the data obtained indicate that 15 to 20 per cent of the phosphate in bone ash is capable of rapid exchange.

The assumption that a *rapid* exchange adsorption exists between a small fraction of the bone surfaces and the inorganic phosphate of blood explains the rapid, initial uptake of the tracer. This assumption alone cannot account for a continued high specific activity of the bone while the specific activity of the blood inorganic phosphorus is diminishing rapidly. We should expect the specific activity-time curve of the fraction of bone which is in rapid equilibrium with blood to be like that represented by Curve II in Fig. 3. The curve should pass through a maximum at which point the blood-bone specific activities are identical. Thereafter, the curve for bone should follow the curve for blood, but above it (13). Correspondingly, if the rest of the bone contained no tagged phosphate, the specific activity-time curve for whole bone would be like that represented by Curve IV, Fig. 3, in which each point is 5 per cent of the corresponding ordinate of Curve II. The experimental data (Curve III) in general fall above the predicted values.

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[3] Data on the growth of femur in similar rats were available. The figure of 8 per cent is based on two assumptions; (*a*) all femur growth took place in the femur end, and (*b*) the phosphate newly acquired had the same average specific activity coefficient as did the inorganic phosphate of plasma during the time interval considered.

[4] By the term "exchange adsorption," a *net* change in the amounts of phosphate in the soluble and insoluble phases is neither implied nor excluded.

There are two possible explanations for the observed data represented by Curve III. Either (a) the initial exchange is irreversible, in which case the rate of accretion of bone salt would be far greater than is actually observed as due to growth (*vide supra*), or, more likely, (b) the bulk of the bone is
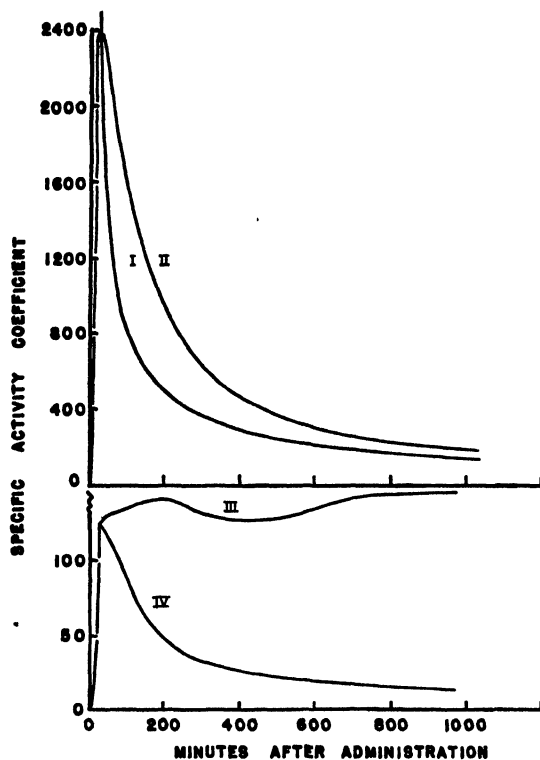
FIG. 3. Curves showing the activities observed for blood and femur ends and the activities expected if only a small portion of bone were in rapid equilibrium with the circulating fluids. Curve I is a plot of the observed activities of the inorganic phosphate of blood. Curve II is a plot of the theoretical values expected (13) for the portion of bone in rapid equilibrium with blood. Curve III represents the observed activities for the phosphate of femur ends. Curve IV is the expected curve if 5 per cent of the phosphate in the femur ends were in rapid equilibrium with blood.

capable of equilibrating slowly with the surface phosphate of high activity, resulting in slow diffusion of active phosphate into the less accessible regions of the bone. Thus, the surfaces of the crystals of bone salt adjacent to the circulating fluid are in very rapid equilibrium with the circulating inorganic phosphate, and the equilibrium becomes progressively slower (with respect to circulating inorganic phosphate) with increasing distance from the circu-

lation. We would suggest that the difference between exchangeable and non-exchangeable phosphate is not the result of differences in physiological function but rather in location. Only phosphate located in the surfaces of the apatite crystals can exchange. Further, it is not necessary to conclude that the exchangeable fraction varies in magnitude in different bones. This concept leads to the conclusion that all calcified structures contain the same amount of exchangeable phosphate per unit weight of bone salt (provided the apatite crystals are similar in size). Differences in uptake of radioactive phosphorus between different bones can be attributed to differences in *rate* of exchange. The rate of exchange will be dependent upon the relative number and size of the blood vessels, marrow spaces, Haversian systems, etc., present in the structure under consideration. It follows that any condition which affects the general circulation (hemoconcentration, altered blood volume, capillary damage, etc.) will affect the uptake of radioactive phosphate by bone and that an altered uptake by bone does not necessarily indicate a direct effect on bone metabolism under such circumstances.

It is evident that whatever the physicochemical process involved, the simple exchange of radioactive phosphate between bone salt and blood is of such a large magnitude that radioactive phosphorus is an unreliable indicator of bone growth in experiments of short duration.

The magnitude of the exchange of radioactive phosphate and the rapidity with which exchange takes place serve to emphasize the dynamic behavior of the calcified tissues. Bone cannot be considered to be a stable system in which the inorganic phase is inert. Rather, the apparent stability of the skeletal system can be attributed only to an extremely efficient control of the inorganic composition of the circulating fluids. Indeed, there are numerous references in the clinical literature to severe disturbances of fluid balance, acid-base balance, etc., which may result in very rapid mobilization of calcium and phosphorus from the bone.

Since the level of isotopic phosphate in blood is largely determined by the rate of exchange between bone and the circulating fluids, it follows that the rate of appearance of labeled phosphate in *all* metabolic compounds containing phosphate such as phospholipides, nucleoprotein, and phosphate intermediates in carbohydrate metabolism, etc., will be influenced by the exchange process.

SUMMARY

There is an initial, rapid entrance of labeled phosphate into the bones following administration of a trace amount of the isotope. Most of this isotopic phosphate is temporarily "fixed" in the bones by an ionic exchange between the fluids and the apatite crystals. The mechanism of this ex-

change is discussed. The magnitude of the exchange seriously detracts from the usefulness of labeled phosphate as a tracer in studies of bone deposition.

Severe choline deficiency in the albino rat did not alter the uptake of isotopic phosphate by the femur to any marked degree. A mild deficiency showed no effect.

A comparison of the average ash weights of the femurs and of the tibias from choline-deficient rats with corresponding tissues from their control animals shows that these bones have grown normally in spite of the general inanition.

The authors are pleased to acknowledge the able assistance of Miss R. Phyllis Fox and are grateful for profitable discussions with Dr. H. C. Hodge and Miss Fox. We are indebted to Miss Betty Mulryan for technical assistance and to W. Schultz and the R. J. Strasenburgh Company, Rochester, New York, for the preparation of guanidoacetic acid.

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EFFECT OF VITAMIN B₆ DEFICIENCY ON THE ABILITY OF RATS AND MICE TO CONVERT TRYPTOPHANE TO N¹-METHYLNICOTINAMIDE AND NICOTINIC ACID*

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Recent studies show that the albino rat, pig, horse, and cotton-rat can convert dietary tryptophane to nicotinic acid or its methylated derivative, N¹-methylnicotinamide (1–4). The ingestion of high amounts of tryptophane, either as the free amino acid or as casein, accentuates a deficiency of vitamin B₆ in the rat and more strikingly so in the mouse (5, 6). The amount of xanthurenic acid excreted by the vitamin B₆-deficient animal is markedly increased when high levels of tryptophane are fed (5, 6). Concurrently, there is a much more rapid depletion of hepatic vitamin B₆ (7). These findings suggested that a vitamin B₆ deficiency may affect the metabolic conversion of tryptophane to nicotinic acid and N¹-methylnicotinamide. Experiments were carried out to study this effect with the rat and with the mouse.

* **EXPERIMENTAL**

Experiments with Rats—Weanling male and female albino rats, 35 to 50 gm. in weight, were used in this study. Group I received the control diet consisting of sucrose 67 per cent, purified casein 24 per cent, Salts IV (8) 4 per cent, and corn oil 5 per cent. The following amounts of vitamins were added per 100 gm. of diet; pyridoxine 250 γ, thiamine 250 γ, riboflavin 300 γ, calcium pantothenate 2 mg., inositol 100 mg., choline 100 mg., and *p*-aminobenzoic acid 30 mg. Vitamins A and D were given orally. Pyridoxine was omitted from the ration for Goup II. In the first experiments five animals were used in each group. Food and water were supplied *ad libitum*.

After the rats had been fed these diets for 3 weeks, they were placed in metabolism cages fitted with outside feeders and urine collections were made. Toluene and 2.5 ml. of 2 N hydrochloric acid were added to the

receiving flasks as preservatives. Urine collections were made for two periods of 3 days each. The amount of food consumed was measured during the collection periods. 100 mg. of *dl*-tryptophane were then fed per rat per day for a period of 3 days. The tryptophane was mixed thoroughly with a weighed amount of the diet. The food intake for each group was restricted slightly in order to assure complete consumption of the tryptophane. Nicotinic acid was determined in the urine with *Lactobacillus arabinosus* as the test organism (9) and N^1-methylnicotinamide was determined fluorometrically (10) without hydrolysis of the samples. These determinations were made on the pooled collections for the 3 day periods.
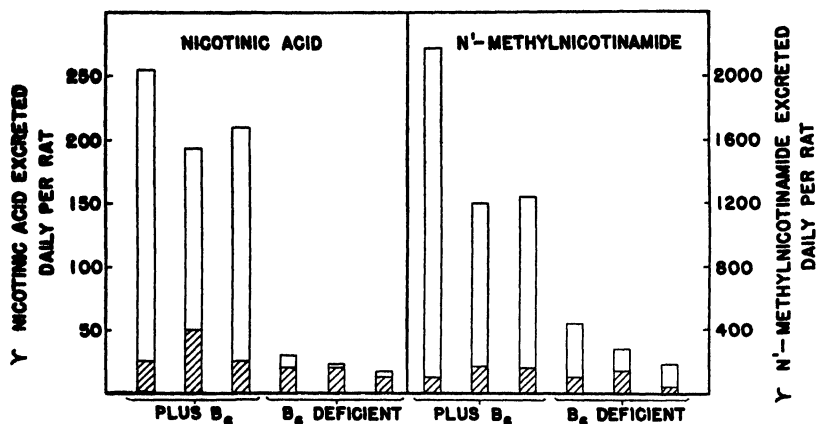
Fig. 1. Effect of feeding tryptophane on the urinary excretion of nicotinic acid and N^1-methylnicotinamide by pyridoxine-supplemented and deficient rats. Shaded areas represent the amounts excreted when the basal diets were fed and the unshaded areas represent the amounts excreted when 100 mg. of *dl*-tryptophane were fed in addition to the basal diets.

This sequence of experimentation was repeated twice with the same groups of animals in order to study the effect of the progressive vitamin B₆ deficiency on the excretion of nicotinic acid and N^1-methylnicotinamide. The results obtained with alternate periods of feeding the basal diets and the same diets plus added tryptophane on the excretion of these two compounds are presented in Fig. 1.

These data show that a vitamin B₆-deficient rat is unable to convert appreciable amounts of the added tryptophane to N^1-methylnicotinamide or nicotinic acid. Approximately 5 times as much N^1-methylnicotinamide was excreted per day in each of the three tests when tryptophane was fed to pyridoxine-supplemented animals as was excreted by the deficient animals (2190, 1190, and 1220 γ per rat per day in contrast to 435, 278, and 187 γ per rat per day). The group receiving pyridoxine and added trypto-

phane excreted 254, 192, and 210 γ of nicotinic acid per rat per day, as compared to 30, 21, and 17 γ excreted by the deficient group also receiving added tryptophane. The amounts of N^1-methylnicotinamide and nicotinic acid excreted when no tryptophane was added were slightly lower for the deficient group. It is significant to note that in the 3 day periods following the periods of tryptophane feeding the amounts of N^1-methylnicotinamide and nicotinic acid excreted are very similar to those before the ingestion of tryptophane. This has also been observed for the cotton-rat and horse (3).

The amount of the dietary tryptophane converted to nicotinic acid and N^1-methylnicotinamide on a weight basis was calculated. When the basal diets were fed, an average of 0.54 per cent of the tryptophane was converted to N^1-methylnicotinamide by the pyridoxine-supplemented group and 0.64 per cent by the deficient group. When tryptophane was fed to the pyridoxine-supplemented group, an average of 2.1 per cent conversion to N^1-methylnicotinamide occurred for the three test periods; however, only 0.50 per cent was converted to N^1-methylnicotinamide by the vitamin B_6-deficient animals. An average of 0.3 per cent of the tryptophane was excreted as nicotinic acid when tryptophane was fed to the pyridoxine-supplemented animals and only 0.03 per cent by the deficient animals, as contrasted to 0.14 and 0.11 per cent, respectively, when no tryptophane was added. Thus, not only the actual amounts of N^1-methylnicotinamide and nicotinic acid excreted by the animals receiving pyridoxine were increased when tryptophane was fed, but the percentage of the ingested tryptophane that was excreted was also increased. In these calculations casein was assumed to contain 1.3 per cent tryptophane (11) and the d isomer was disregarded in the calculations (see Rosen et al. (1)).

To confirm these observations, a second series of experiments was carried out. The same diets and experimental procedures were used. Four rats were used per group and urine collections were made after 4 weeks from the start of the experiment. The effect of injecting an equivalent amount of l-tryptophane on the excretion of N^1-methylnicotinamide, nicotinic acid, and of tryptophane was also tested. The amount of apparent free tryptophane excreted was determined microbiologically (12, 13). In these tests 2.5 ml. of a 2 per cent $NaHCO_3$ solution were injected intraperitoneally twice daily during the basal collection period. For the following 2 days, 2.5 ml. of a 2 per cent $NaHCO_3$ solution which contained 10 mg. of l-tryptophane per ml. were injected twice daily. The results for the amounts of N^1-methylnicotinamide and nicotinic acid excreted per day and also per gm. of food consumed are shown in Table I.

The results obtained agree very well with those obtained in the first experiments on the effects of feeding tryptophane to the vitamin B_6-sup-

plemented and the deficient groups. The differences between the two groups are clearly not a reflection of the caloric intake, since on the basis of the excretion per gm. of food a 5- to 10-fold increase was obtained in favor of the pyridoxine-supplemented group when tryptophane was fed. Much smaller amounts of both N^1-methylnicotinamide and nicotinic acid were excreted when tryptophane was injected, as contrasted to when it was fed.

<div align="center">TABLE I</div>

Influence of Vitamin B₆ Deficiency and Administration of Tryptophane on Urinary Excretion of N^1-Methylnicotinamide and Nicotinic Acid by the Rat

Group No.	Dietary regimen	Collection period	N^1-Methylnicotinamide		Nicotinic acid	
			γ per rat per day	γ per gm. food consumed	γ per rat per day	γ per gm. food consumed
		days				
I (+ vitamin B₆)	Basal	5	93	9 3	23 4	2.4
	" + tryptophane	3	1530	170	430	47 7
	Basal	3	181	19 8	43	4.7
	" + tryptophane	3	809	105	180	23 5
	Basal	2	160	18 2	39	4 4
	" + tryptophane*	2	338	38 6	95	10 8
II (no vitamin B₆)	Basal	5	48	9 9	9 9	2 1
	" + tryptophane	3	106	41 2	19 7	7 7
	Basal	3	59	11 8	12 7	2 5
	" + tryptophane	3	224	55.0	34 7	4 3
	Basal	2	88	16 6	12 5	2 4
	" + tryptophane*	2	154	32 2	15 0	3 2

* In these tests, 50 mg. of *l*-tryptophane were given per day by intraperitoneal injection. In all other cases 100 mg. of *dl*-tryptophane were fed daily.

Less than 1 per cent of the injected tryptophane was excreted in the urine. No appreciable difference was noted between the pyridoxine-supplemented and deficient groups. Thus it appears that tryptophane is rapidly metabolized to microbiologically inactive compounds when injected or fed orally as casein (13) both by normal and pyridoxine-deficient rats.

The vitamin B₆-supplemented animals grew at a rate of 20 gm. per week, while the deficient animals grew at a rate of 10 gm. per week for the first 4 weeks. After this period, the latter group reached a plateau in weight.

Experiments with Mice—Experiments similar to those conducted with rats were also conducted with albino mice. In the first experiment animals which weighed 16 to 20 gm. were used. In the second experiment,

weanling mice (7.2 to 9.5 gm.) were used. Four to six mice were in each group and were fed the same diets as described for the rats. Urine collections were made after 2 weeks on experiment and the same procedures for feeding and urine collections were used, except that each mouse received 50 mg. of dl-tryptophane per day. Since the mice scattered considerable food, no attempt has been made to calculate the amounts of N^1-methylnicotinamide and nicotinic acid excreted per gm. of food consumed. Contamination of the urine with the ration does not negate the validity of the nicotinic acid determinations, since none of this vitamin was added to the diet. The results for the two experiments were very similar; therefore the data have been averaged together and are presented in Table II.

TABLE II

Influence of Vitamin B_6 Deficiency and Ingestion of Tryptophane on Urinary Excretion of N^1-Methylnicotinamide and Nicotinic Acid by the Mouse

Expressed as micrograms excreted per mouse per day.

Dietary regimen	Collection period	N^1-Methylnicotinamide		Nicotinic acid	
		Plus vitamin B_6	No vitamin B_6	Plus vitamin B_6	No vitamin B_6
	days				
Basal	5	14 4	14 5	7.9	4 4
" + tryptophane*	3	63 6	36 0	31 0	12 5
"	3	18 7	13 7	12 8	5 2
" + tryptophane*	3	63.0	33 7	45 2	5 0
"	2	25 1	10 6	12.5	4.0
" + tryptophane*	3	84 2	28 3	57.2	4 1

* 50 mg. of dl-tryptophane were fed per mouse per day.

A 3.5-fold increase in the amounts of N^1-methylnicotinamide and nicotinic acid excreted was observed when tryptophane was fed to the mice receiving pyridoxine. The vitamin B_6-deficient animals excreted 2 to 3 times as much N^1-methylnicotinamide, but no increase due to the ingestion of tryptophane was observed in the nicotinic acid values after 4 weeks on experiment. From these data it is apparent that the mouse responded qualitatively like the rat.

That a pronounced vitamin B_6 deficiency was produced was evident from the slow growth during the first 4 weeks (0.4 gm. per week) and a decrease in weight thereafter. The vitamin B_6-supplemented group grew at a normal rate. Qualitatively, a marked increase in the excretion of xanthurenic acid was observed, particularly when tryptophane was fed, which is in accord with the work of Miller and Baumann (5, 6).

DISCUSSION

The present study shows that when tryptophane is fed, vitamin B_6-deficient rats and mice have a greatly reduced ability to convert this amino

acid to nicotinic acid and N¹-methylnicotinamide. The rôle of vitamin B₆ in this metabolic reaction offers additional evidence for its importance in amino acid metabolism. The mechanism by which tryptophane can be converted to nicotinic acid (which is then presumably methylated to some extent by the rat and mouse to form N¹-methylnicotinamide) remains to be elucidated. This transformation apparently can be accomplished by the rat, mouse, pig, cotton-rat, and horse. The relative inability of the vitamin B₆-deficient animal to convert tryptophane to nicotinic acid and its methylated derivative would suggest that xanthurenic acid cannot serve as a precursor of nicotinic acid.

It is not surprising that much smaller amounts of nicotinic acid and N¹-methylnicotinamide were formed when tryptophane was injected, as compared to when the amino acid was fed. Tryptophane is rapidly removed from the blood of both normal and vitamin B₆-deficient rats after injection and returns to a normal level within 1 to 2 hours after injection (13). Since only two injections were made in each 24 hour period, a relatively continuous supply of tryptophane during the period was not achieved, as compared to the situation produced when the compound was fed. The increased excretion of nicotinic acid and N¹-methylnicotinamide after injection indicates that nicotinic acid is formed from tryptophane largely by synthesis in the tissues rather than by microbial synthesis in the intestinal tract. The rapid increase observed in the amounts of nicotinic acid excreted during tryptophane feeding and the rapid return to a normal level following the feeding of tryptophane support this view. This observation has also been reported in a recent paper by Singal et al. (4). Briggs and associates (14) offer evidence that nicotinic acid may be synthesized in the tissues of chicks fed tryptophane, while Krehl et al. (15) suggest that the counteraction of the growth depression due to ingestion of high levels of corn when rats are fed tryptophane may be due to stimulation of nicotinic acid synthesis by tryptophane. Thus the interrelationships of vitamin B₆, nicotinic acid, and tryptophane in the nutrition of rat and mouse demonstrate the complex nature of these metabolic reactions and offer new approaches to further studies on amino acid metabolism. These data suggest that the beneficial effect of pyridoxine therapy for pellagrins may be due in part to its influence on the conversion of ingested tryptophane to nicotinic acid.

SUMMARY

1. The effect of vitamin B₆ deficiency on the conversion of tryptophane to nicotinic acid and N¹-methylnicotinamide has been determined with the rat and mouse.

2. When 100 mg. of dl-tryptophane were fed in addition to the basal

ration, rats fed pyridoxine excreted 810 to 2190 γ of N^1-methylnicotinamide, while deficient animals excreted 180 to 435 γ per day. When the basal diets were fed, the two groups excreted 95 to 185 and 45 to 140 γ per day of N^1-methylnicotinamide, respectively.

3. The excretion of nicotinic acid when tryptophane was fed ranged from 95 to 430 γ per rat per day for the vitamin B_6-supplemented group and only 16 to 35 γ for the vitamin B_6-deficient group. When the basal diets were fed, 23 to 50 γ and 10 to 24 γ of nicotinic acid were excreted by the two groups respectively.

4. When tryptophane was injected, a 100 per cent increase in the excretion of N^1-methylnicotinamide and nicotinic acid was observed for the animals fed pyridoxine.

5. Similar effects were obtained when tryptophane was fed to pyridoxine-supplemented and deficient mice.

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THE COMPARATIVE UTILIZATION OF PTEROYLGLUTAMIC ACID AND PTEROYLTRIGLUTAMIC ACID BY CHICKS ON A PURIFIED DIET

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The isolation, identification, and synthesis of pteroylglutamic acid from liver have been described; simultaneously the isolation was reported of a compound, "fermentation *Lactobacillus casei* factor," which upon mild anaerobic alkaline hydrolysis yielded pteroylglutamic acid and 2 equivalents of glutamic acid (1). This compound has been termed pteroyltriglutamic acid (2).

Studies with chicks on a purified diet deficient in pteroylglutamic acid (3) indicated that pteroyltriglutamic acid, when fed at a level of 0.5 part per million of diet, was not markedly effective in promoting growth or preventing anemia. The addition of the lactone of either 2-methyl-3-hydroxy-4-hydroxymethyl-5-carboxypyridine (5-pyridoxic acid lactone) or 2-methyl-3-hydroxy-4-carboxy-5-hydroxymethylpyridine (4-pyridoxic acid lactone) together with pteroyltriglutamic acid produced a marked gain in weight over the controls and was completely effective in the prevention of anemia as measured by the hemoglobin level at 3 weeks. In another communication (4) it was reported that pteroylglutamic acid was fully effective for the prevention of anemia without the addition of the lactone. It was suggested (4) that the lactone of 4-pyridoxic acid functioned in an enzyme system required for the breakdown of "folic acid conjugates" and for the setting free of "folic acid" (pteroylglutamic acid).

However, other experiments have indicated that the chick is able to utilize pure pteroylheptaglutamic acid ("vitamin B_c conjugate") as efficiently as pteroylglutamic acid ("vitamin B_c") (5) and that pteroyltriglutamic acid is utilized just as well as pteroylglutamic acid (6) for hemoglobin formation in chicks on purified diets without the addition of pyridoxic acid lactone. These experiments were made with basal diets differing from that employed by the Cornell group (3). The present investigation was undertaken in an attempt to repeat more closely the experimental conditions employed elsewhere (3).

EXPERIMENTAL

The basal diet was identical in composition with that described elsewhere (3) and consisted of starch, washed casein, gelatin, salt mixture, soy bean

oil, cellophane, and fortified fish liver oil supplemented with pure vitamins and with succinylsulfathiazole, 1 per cent. The only known difference was that the soy bean oil used in the present investigation did not contain phosphoric acid as an antioxidant.

Day-old New Hampshire chicks were placed in electrically heated battery brooders and were fed the experimental diets immediately. Ten chicks were used in each group. The diets were mixed in small quantities at frequent intervals and were kept in a refrigerator. Acid-water-washed casein was supplied by Dr. M. L. Scott.

The following supplements, per kilo of basal diet, were used in the first experiment: Group 1, none; Group 2, 0.5 mg. of pteroyltriglutamic acid; Group 3, 1.0 mg. of pteroyltriglutamic acid; Group 4, 0.5 mg. of pteroyltrig'utamic acid + 1.0 mg. of 4-pyridoxic acid lactone; Group 5, 1.0 mg. of pteroyltriglutamic acid + 1.0 mg. of 4-pyridoxic acid lactone; Group 6, 0.67 mg. of pteroylglutamic acid.

The purity of the pteroyltriglutamic acid was estimated by measurement of its absorption of ultraviolet light in aqueous solution at 365 mμ. The preparation was approximately 95 per cent pure. It was obtained from a fermentation product (1). The pteroylglutamic acid preparation was 90 per cent pure, and 0.75 mg. was used to correspond to 0.67 mg. of the pure substance. This level was equivalent on a molar basis to 1.06 mg. of pteroyltriglutamic acid. Pteroyltriglutamic acid and pteroylglutamic acid were dissolved in water at a concentration of 0.1 mg. per ml., and the lactone at a concentration of 1.0 mg. per ml. The solutions were incorporated in the diets. Hemoglobin determinations, erythrocyte counts, and hematocrit measurements were made with blood from a wing vein. The results of the experiment are summarized in Table I.

Three cases of perosis were noted in seven surviving birds in Group 1 at 20 days. One case of perosis was simultaneously noted in each of Groups 2 and 4. Pteroyltriglutamic acid appeared to be fully effective in preventing anemia, even when fed at a level of 0.5 mg. per kilo of diet, which corresponded to only 0.32 mg. of pteroylglutamic acid.

The possibility remained that at a lower level pteroyltriglutamic acid, even if fully utilized, might be only partially effective in preventing anemia and that a condition more sensitive for measuring the supplementary effect of the 4-pyridoxic acid lactone might thus be established. Accordingly a second experiment was carried out 3 months later. The following supplements, per kilo of diet, were used: Group 7, none; Group 8, 0.3 mg. of pteroyltriglutamic acid; Group 9, 0.3 mg. of pteroyltriglutamic acid + 1.0 mg. of 4-pyridoxic acid lactone; Group 10, 0.2 mg. of pteroylglutamic acid; Group 11, 0.65 mg. of pteroylglutamic acid.   .

The results are summarized in Table II. The level of pteroylglutamic

acid fed to Group 10 was stoichiometrically approximately equivalent to the level of pteroyltriglutamic acid fed to Groups 8 and 9. Group 11 as a "positive control" group received a level of pteroylglutamic acid in excess of the requirement for growth and hemoglobin formation; this level is in

TABLE I

Response of Chicks to Pteroyltriglutamic Acid, with and without 4-Pyridoxic Acid Lactone, and to Pteroylglutamic Acid

Group No	Average weight at			Hemoglobin at		Erythrocyte count	Mean corpuscular volume $\times 10^{-2}$
	1 day	21 days	28 days	21 days	28 days	28 days	28 days
	gm.	gm	gm	gm per cent	gm per cent	millions per c mm.	cu microns
1	43	82*	74†	5.0	1.3	0.50	‡
2	42	222	299	8.7	8.5	2.50	1.02
3	44	252	344	8.7	8.5	2.65	0.97
4	43	235	331	8.9	8.5	2.43	1.05
5	40	247	338	8.8	8.5	2.59	1.00
6	42	244	335	8.6	8.6	2.73	0.92

* Five survivors.
† One survivor.
‡ Not determined.

TABLE II

Results of Second Experiment in which Insufficient Levels of Pteroyltriglutamic Acid and Pteroylglutamic Acid Were Fed in Groups 8, 9, and 10

Group No.	Average weight at			Hemoglobin at		Erythrocyte count	Mean corpuscular volume $\times 10^{-2}$
	1 day	21 days	28 days	21 days	28 days	28 days	28 days
	gm.	gm.	gm.	gm. per cent	gm per cent	millions per c mm.	cu. microns
7	45	102	122	5.7	5.0	1.12	1.23
8	44	158	211	8 2	9 0	2.62	0.91
9	44	135	191	8.2	8.2	2.16	0.97
10	44	144	190	7.1	8.1	2.14	1.00
11	45	174	248	8.9	9.5	2.87	0.93

the neighborhood of 0.4 to 0.5 mg. The growth of the chicks in the second experiment was markedly slower than in the first experiment. It may be speculated that this was due to a diminished carry over of some unidentified factor from the maternal diet in the second experiment. Again, the utilization of pteroyltriglutamic acid appeared to be complete as com-

pared with the corresponding level of pteroylglutamic acid, and the addition of 4-pyridoxic acid lactone appeared to have no effect. Three cases of perosis at 28 days were observed in Group 7, three in Group 8, one in Group 9, two in Group 10, and none in Group 11.

DISCUSSION

In a single experiment involving a comparison between two groups of six chicks each, it was noted that 4-pyridoxic acid had a supplementary effect on growth and hemoglobin formation when added to a purified diet containing pteroyltriglutamic acid. No supplementary effect on yeast concentrate was obtained by adding 4-pyridoxic acid (7).

In another investigation (8) it was noted that 4-pyridoxic acid lactone had no supplemental effect on the action of a suboptimal amount of "vitamin B_c" (pteroylglutamic acid) in promoting growth, feathering, and hemopoiesis in the chick. This result is in agreement with the report published elsewhere (4) and has no bearing on the claim that the lactone is concerned with the breakdown of pteroylglutamic acid conjugates.

The present series of experiments fails to confirm the results published elsewhere (3), although an attempt was made to duplicate the diet. It may be noted, however, that New Hampshire chicks were used in the present investigation, while white Leghorn chicks were used by the Cornell group. Another possibility is that pyridoxic acid lactone was present in some constituent of the diet or was carried over from the eggs into the newly hatched chicks.

SUMMARY

1. Chicks were found to utilize pteroyltriglutamic acid and pteroylglutamic acid equally well on a molar basis for growth and the prevention of anemia.

2. The addition of 4-pyridoxic acid lactone had no measurable effect on the utilization of pteroyltriglutamic. acid under the conditions of the experiments.

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THE OXIDATION OF PHOSPHORYLATED AND NON-PHOS-PHORYLATED SUGARS BY MAMMALIAN LIVER*

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In 1928 Müller (1) reported the presence of a new enzyme, a glucose oxidase, in press-juice of *Aspergillus niger*. He subsequently suggested that it might be a dehydrogenase (2); he later showed that it catalyzed the oxidation of glucose to gluconic acid (3). Harrison in 1931 obtained glucose dehydrogenase from acetone-dried mammalian liver by saturating a water extract with ammonium sulfate (4). The product of the oxidation was shown to be gluconic acid (5) and the cytochrome system a hydrogen carrier (6). Müller then resumed his work with glucose dehydrogenase and demonstrated the decolorization of methylene blue in the presence of a catalytic enzyme preparation from ox liver when galactose and xylose were present as substrates (7). Breusch (8) has more recently reported the existence of a D-arabinose dehydrogenase, a glyceraldehyde dehydrogenase, and a glycoaldehyde dehydrogenase in cat liver, and the probable existence of a D-erythrose dehydrogenase in the same tissue.

Several dehydrogenases attacking phosphorylated derivatives of hexoses have been reported. Warburg and Christian (9) found an enzyme in erythrocytes that oxidizes glucose-6-phosphate when methylene blue is the carrier. The name *Zwischenferment* was given by them (10) to the same or a similar hexose monophosphate dehydrogenase obtained from yeast which oxidizes glucose-6-phosphate to phosphogluconic acid. Harrison (11) has indicated the presence of a hexose diphosphate dehydrogenase in muscle and liver which operates in the presence of methylene blue.

The experiments reported herein verify the existence of enzymes in lamb liver that are capable of oxidizing D-glucose, D-arabinose, D-xylose, D-lyxose, D-glucose-6-phosphate, fructose-6-phosphate, and fructose-1,6-phosphate when methylene blue and diphospho- and triphosphopyridine nucleotide are added to a partially purified preparation.

EXPERIMENTAL

Preparations

Lyophilization of Tissues—The liver was obtained from the killing floor of a slaughter-house, and was usually placed in the freezing compartment of a refrigerator for temporary storage within 1 hour after removal from the

animal. In the next half hour the tissue was ground, frozen, and connected to the lyophilizer. Lyophilization of 100 gm. of fresh tissue was complete in from 6 to 8 hours. The dry liver was then stored in a vacuum desiccator over P_2O_5 at 5°. Lyophilization was preferred to the acetone drying recommended by Harrison (4) because the latter method offers even greater possibility for the denaturation of unidentified enzymes.

Harrison's Glucose Dehydrogenase and Coenzyme—These were prepared according to Harrison (12) with this exception: lyophilized liver was used instead of acetone-dried liver.

Partially Purified Lamb Liver Dehydrogenases. Preparation A—On the 1st day 60 to 80 gm. of thoroughly dried lyophilized lamb liver were ground' in a mortar to a very fine powder, mixed in the same container with 4 volumes of ice-cold distilled water, and extracted in an ice bath for 30 minutes with occasional stirring. The mixture was centrifuged for 15 minutes at 3800 R.P.M. in 50 ml. celluloid tubes. The supernatant was poured off through cheese-cloth and the residue reextracted twice more with 2 volumes of cold distilled water, with stirring, for 15 minutes. The combined supernatants were dialyzed overnight against distilled water at 5°.

On the 2nd day the protein solution was centrifuged for 15 minutes at 3800 R.P.M. The pH was usually found to be between 6.0 and 6.5 and was taken to 5.7 with N HCl. Following centrifugation at 3800 R.P.M., the pH was adjusted to 7.0 with N NaOH. 21 gm. of $(NH_4)_2SO_4$ per 100 ml. were added (30 per cent saturated), and the precipitate centrifuged off at 3800 R.P.M. and discarded. 14 gm, of $(NH_4)_2SO_4$ per 100 ml. (original volume) were added to the supernatant (50 per cent saturated) and the precipitate centrifuged down (supernatant discarded) and dissolved in 50 ml. of cold distilled water. The proteins were again separated by adding 17.5 gm. of $(NH_4)_2SO_4$ (50 per cent saturated) and by centrifuging for 15 minutes at 3800 R.P.M. The precipitate was dissolved in 50 ml. of cold distilled water and dialyzed overnight against 10 per cent methanol at 5°.

On the 3rd day the solution was centrifuged for 15 minutes at 3800 R.P.M. and the supernatant mixed with 25 ml. of an $Al(OH)_3$ gel (for the preparation, see below). Following centrifugation the supernatant was treated with successive portions of $Al(OH)_3$ gel until nearly colorless or until 50 ml. of the gel had been used. The proteins were eluted with successive 20 ml. portions of 0.1 N NH_4OH which contained 17.5 gm. of $(NH_4)_2SO_4$ per 100 ml. (25 per cent saturated) until the gel was nearly colorless or until 80 ml. had been used. The combined eluates were dialyzed overnight against water at 5°.

On the 4th day the solution was lyophilized in 5 to 8 hours and the resulting dry protein stored in a vacuum desiccator over P_2O_5 at 5°. The yield ranged between 1.5 and 2.0 gm.

Preparation B—The procedure was as for Preparation A, with this excep-

tion: the protein was dialyzed against distilled water instead of against 10 per cent methanol at the end of the 2nd day.

Preparation C—The procedure was as in the case of Preparation A with these exceptions: (1) special precautions were taken to keep the preparation cold at all stages except during the centrifugations when refrigeration was not available; (2) the 60 to 80 gm. of lyophilized lamb liver were homogenized in a Waring blendor for 1 minute with 4 volumes of cold distilled water instead of being ground in a mortar; (3) the protein was dialyzed against distilled water at the end of the 2nd day instead of against 10 per cent methanol.

Al(OH)₃ gel was prepared according to the method of Willstätter and Kraut (13), 24 mg. of $Al(OH)_3$ per ml.

TPN and DPN[1] were prepared according to the method of Warburg and Christian (14).

Hexose monophosphate was prepared according to the method of DuBois and Potter (15).

Fructose-6-phosphate—To 10 gm. of the commercial fructose-1,6-diphosphate[2] used in these experiments were added 100 ml. of 0.1 N H_2SO_4, and the solution was heated in a water bath for 15 minutes at 100°. The reaction mixture was then cooled, put into centrifuge tubes, and precipitated with 2 volumes of 95 per cent ethanol. The precipitate was centrifuged down (supernatant discarded), dissolved in 100 ml. of distilled water, and the solution filtered. The salt was precipitated by the addition of 2 volumes of 95 per cent ethanol, and then once more dissolved and reprecipitated. The preparation was finally washed successively with 66 per cent ethanol, 95 per cent ethanol, acetone, and ether. The yield was approximately 6.5 gm. of the Ca salt.

Glucose-6-phosphate was prepared according to the method of Levene and Raymond (16) with a modification of the first step; that is, the preparation of the diacetone glucose. 2 liters of acetone (U. S. P.) were mixed with 20 ml. of H_2SO_4 (density, 1.84, reagent grade) and to the mixture were added 250 gm. of anhydrous $CuSO_4$ and 250 gm. of anhydrous D-glucose (c.p.). The suspension was stirred continuously for 48 hours at 5°. This is a departure from the usual method, which calls for stirring at room temperature for 24 hours. The suspension was filtered through Whatman No. 5 paper with suction and the clear filtrate neutralized with approximately 250 gm. of $Ca(OH)_2$. The resulting suspension was filtered with suction, but without the previous addition of charcoal. There was no evidence of carbonization or of caramelization. The clear filtrate was concentrated to a yellowish solid under reduced pressure and at an elevated bath temperature

[1] DPN, diphosphopyridine nucleotide; TPN, triphosphopyridine nucleotide.
[2] Schwarz Laboratories, Inc.

(approximately 50–55°, with an H_2O pump for reducing the pressure). It was unnecessary next to extract repeatedly with heptane and to decolorize with charcoal, since the resulting solid was completely soluble in boiling heptane and since, as stated before, it had not carbonized or caramelized. The yield was approximately 130 gm., or 30 gm. less than the yield in the original method.

TABLE I

Oxygen Consumption of Rabbit Liver Slices As Influenced by Addition of Various Substrates

Experiment No.	Substrate	Oxygen consumption	
		60 min.	120 min.
		c.mm.	*c.mm.*
1	None	144	252
	D-Glucose	148	255
	D-Galactose (Eastman)	157	265
	D-Arabinose	152	262
	D-Lyxose	152	257
	D-Xylose (Eimer and Amend)	146	248
2	None	123	206
	D-Glucose	128	213
	D-Mannose	130	218
	Fructose	127	210
	L-Arabinose	120	197
3	None	151	272
	D-Glucose	156	271
	D-Glucose-6-phosphate	153	270
	Fructose-6-phosphate	150	263
	Fructose-1,6-diphosphate	179	336

Tissue, 250 mg. of rabbit liver slices; electrolyte-buffer solution, 1.75 ml. of a NaCl, KCl, $MgSO_4$, and sodium phosphate solution at pH 7.32 (Krebs (23)); substrate, 0.25 ml. of 5.4 per cent hexoses, or 4.5 per cent pentoses; gas phase, oxygen; center well, 10 per cent NaOH; rate of shaking, 120 per minute; temperature, 37.5°.

Liver Slices

Investigators who have studied the oxidation of simple sugars by mammalian liver slices have, for the most part, found no appreciable increase in the oxygen consumption. Glucose when added to rat liver (17–21) or to guinea pig liver (22) has no effect. Whereas galactose (21) and arabinose and xylose (19) have no effect on the oxygen uptake of rat liver, fructose has been reported by Kisch (19) and Marsh (21) to increase the oxygen consumption of normal rat liver slices, although their results are in disagreement with those of Dickens and Greville (17) who find that brain cortex, testis, and kidney are stimulated, but not liver.

In the liver slice experiments, shown in Table I, the oxygen consumption

by 250 mg. of rabbit liver slices suspended in an isotonic electrolyte-buffer solution at pH 7.32 was essentially unchanged by the addition of D-glucose,[3] D-galactose,[4] D-arabinose,[4] D-lyxose,[4] D-xylose,[5] D-mannose,[5] fructose,[4] L-arabinose,[4] D-glucose-6-phosphate, and fructose-6-phosphate. The only substrate that appeared to prevent in part the decrease in the rate of oxygen consumption was fructose-1,6-diphosphate.[2] It seems likely that the glucose derived by hydrolysis from the glycogen contained in the liver was supplying a sufficient amount of a preferred substrate and that, therefore, added substrates were without effect.

H_2O Extracts of Lyophilized Lamb Liver

In view of the fact that glucose dehydrogenase is water-soluble (1, 4), it was decided first to investigate the oxidation of these sugars by water extracts of lyophilized lamb liver.

The results are presented in Table II. D-Galactose (Eastman), D-galactose (Eimer and Amend), D-mannose, D-glucose-6-phosphate, and fructose-6-phosphate seem to have a small stimulating effect, whereas fructose-1,6-diphosphate definitely increases the uptake of oxygen above that of the substrateless control. The failure of D-glucose to increase the oxygen uptake might be explained on the basis that the hydrolysis of glycogen in the liver or in the water extract has already produced sufficient glucose to saturate the enzyme system.

$(NH_4)_2SO_4$ Precipitates of H_2O Extracts of Lyophilized Liver

Harrison (12) made his second glucose dehydrogenase preparation by saturating the water extract to 50 per cent with $(NH_4)_2SO_4$ and found that the resulting proteins were quite reactive toward glucose. This was also the method used by Müller (7) who found that in addition to D-glucose, D-galactose and D-xylose were oxidized; D-mannose and L-arabinose were not. The final concentration of sugar in his Thunberg tubes ranged from 0.125 to 0.5 M. The concentrations were very much higher than the concentration used by Breusch (8) who added 1 mg. of substrate to 3 ml. of fluid to make a final concentration of approximately 0.002 M. Breusch found that only D-arabinose was oxidized and not L-arabinose, D-mannose, D-ribose, D-xylose, or D-galactose.

Early results that we obtained, using Harrison's enzyme and coenzyme preparations and a final concentration of sugar of 0.225 M, are presented in Table III. It would appear that D-glucose, D-xylose, D-galactose (Eimer and Amend), and hexose monophosphate are oxidized, whereas L-arabinose, D-mannose, and D-ribose are not.

[3] Merck and Company, Inc., C.P., anhydrous.
[4] Eastman Kodak Company.
[5] Eimer and Amend, C.P.

TABLE II

Effect of Various Substrates on Oxygen Uptake by Water Extract of Lyophilized Lamb Liver

Experiment No.	Substrate	Oxygen consumption	
		60 min.	120 min.
		c.mm.	*c.mm.*
1	None	109	161
	D-Glucose	106	163
	D-Galactose (Eastman)	123	181
	D-Galactose (Eimer and Amend)	129	193
	D-Mannose	121	179
	Fructose	115	170
2	None	118	168
	D-Glucose	115	174
	D-Arabinose	117	176
	L-Arabinose	116	176
	D-Xylose	111	163
	D-Lyxose	109	168
3	None	110	165
	D-Glucose	110	168
	D-Ribose (Schwarz)	112	173
	D-Glucose-6-phosphate	132	205
	Fructose-6-phosphate	133	199
	Fructose-1,6-diphosphate	156	232

Total volume, 2.5 ml ; extract, 0.5 ml.; preparation, 10 gm. of lyophilized lamb liver, 40 ml. of H_2O; 30 minutes at 0°; centrifuged, decanted; 20 ml. of H_2O to residue; 15 minutes at 0°; decanted; total volume in three experiments, 34, 37, and 37 ml., respectively; buffer, 1.0 ml. of 0.25 M phosphate at pH 7.35; substrate, 0.25 ml. of a 1 M solution; center well, 10 per cent NaOH; gas phase, oxygen; rate of shaking, 100 per minute; temperature, 38°.

TABLE III

Decolorization of Methylene Blue by Harrison's Enzyme-Coenzyme in Presence of Various Sugars

Substrate	Decolorization time	Substrate	Decolorization time
	min.		*min.*
None	>60	L-Arabinose	>60
D-Glucose	7	D-Mannose	>60
D-Xylose	8	D-Ribose	>60
D-Galactose (Eimer and Amend)	35	Hexose monophosphate	8

Total volume, 1.75 ml.; protein, 0.4 ml., equivalent to 0.4 gm. of lyophilized lamb liver, prepared according to Harrison (12); final dry preparation dissolved in water, insoluble portion centrifuged off and discarded, and the pH adjusted to 7.4; coenzyme, 0.4 ml., equivalent to 0.4 gm. of liver, prepared according to Harrison; substrate, 0.2 ml. of a 2 M solution; methylene blue, 0.25 ml. of a 1:5000 solution; water, 0.5 ml.; temperature, 38°.

Partially Purified Dehydrogenase Preparations

The two partially purified enzyme preparations of lyophilized lamb liver, Preparations A and B, which were obtained by successively adjusting pH, fractionating with $(NH_4)_2SO_4$, and adsorbing on $Al(OH)_3$ gel, are undoubtedly mixtures of several dehydrogenases and of other enzymes as well. No special attempt was made to obtain a pure or electrophoretically uniform preparation. It was hoped, rather, to free the dehydrogenases of all the substrates and coenzymes and of most, if not all, of the flavoprotein which might mediate between the added coenzymes DPN and TPN and the added oxygen carrier, methylene blue. The preparations were certainly free of

TABLE IV

Effect of Varying Protein Concentration

Protein per Thunberg tube	Decolorization time	
	Methylene blue	2,6-Dichlorophenol indophenol
mg.	*min.*	*min.*
2 (1.3)*	16	5.5
4 (2.6)	11	2
6 (3.9)	8	1.5
8 (5.2)	7	1

Total volume, 2.05 ml.; protein, 0.4 ml., containing the water-soluble portion of from 2 to 8 mg. of Preparation A after centrifuging off the insoluble proteins; coenzyme, 0.4 ml. of a 50 mg per cent solution of DPN in H_2O; substrate, 0.2 ml of a 1 M solution of D-glucose; buffer, 0 5 ml. of 0.25 M phosphate at pH 7.4; oxidation-reduction indicator, 0.25 ml. of a 1:5000 solution of either dye in H_2O; water, 0.3 ml.; temperature, 38°.

* Approximately 65 per cent goes into solution.

substrate, since without glucose, but with added DPN, TPN, and methylene blue, the oxygen consumption for a 2 hour period averaged only 10 c.mm.

The first series of experiments with these partially purified preparations was carried out in Thunberg tubes with Preparation A. The effect of protein concentration is shown in Table IV. Both methylene blue and 2,6-dichlorophenol indophenol were used as oxidation-reduction indicators. In this and in subsequent experiments with Preparation A the amount of the preparation that dissolved in distilled water averaged approximately 65 per cent of the weighed protein. The results indicate a typical response and the values if plotted would give a hyperbolic curve. The protein concentration chosen for the Thunberg experiments to follow was 2 mg. per tube.

The next experiment was to test the effect of varying the glucose concentration on the decolorization time of both methylene blue and 2,6-dichloro-

phenol indophenol (Table V). The final glucose concentration in the Thunberg tubes ranged from 0.195 to 0.003 M. In the presence of 0.098 M final concentration of glucose, methylene blue was decolorized in 8.5 minutes and 2,6-dichlorophenol indophenol in 3.5 minutes, whereas in the presence of 0.006 M final concentration of glucose (6.1 per cent of 0.098 M) methylene blue was decolorized in 21 minutes and 2,6-dichlorophenol indophenol in 18 minutes. In subsequent tests any substance which at a 0.098 M final substrate concentration decolorizes methylene blue in 21 minutes or less and 2,6-dichlorophenol indophenol in 18 minutes or less would have to be considered oxidized, or would have to contain impurities equivalent to 6 per cent or more of D-glucose.

TABLE V

Effect of Varying Glucose Concentration

Final glucose concentration	Decolorization time	
	Methylene blue	2,6-Dichlorophenol indophenol
M	*min.*	*min.*
0.195	7	3
0.098	8.5	3.5
0.049	9	4
0.024	11.5	5
0.012	15	7
0.006	21	18
0.003	28	37

Total volume, 2.05 ml.; protein, 0.4 ml., containing the water-soluble portion from 2 mg. of Preparation A after centrifuging off the insoluble proteins; approximately 1.30 mg. goes into solution; substrate, 0.2 ml. of a 2 M to M/32 solution of D-glucose; coenzyme, buffer, oxidation-reduction indicator, water, and temperature as in Table IV.

In Table VI we see the effect of various substrates on the decolorization time of the two indicating dyes. The sugars which are themselves oxidized or which may contain impurities equivalent to 6 per cent or more of D-glucose are D-arabinose, D-lyxose, D-xylose (Eastman), D-xylose (Eimer and Amend), and D-glucose itself, of course. With 2,6-dichlorophenol indophenol as an indicator, the two D-galactoses are slowly oxidized, but this might well be due to impurities since decolorization occurs so slowly.

The second series of experiments was performed with Preparation B and the reactions were followed in Warburg vessels. Both DPN and TPN were added in phosphate buffer (pH 7.4), and methylene blue was added to serve as oxygen carrier. The protein concentration which on the addition of 0.25 ml. of 1 M D-glucose (final concentration 0.050 M) was found to cause an oxygen consumption of about 100 c.mm. in 2 hours was 20 mg. per vessel

TABLE VI

Effect of Various Substrates on Decolorization Time

Substrate	⌒ Decolorization time	
	Methylene blue	2,6-Dichlorophenol indophenol
	min.	*min.*
None	120	61
D-Glucose	10	2
D-Arabinose	20	19
D-Galactose (Eimer and Amend)	39	21
D-Galactose (Eastman)	54	29
D-Lyxose	20	51
D-Xylose (Eastman)	21	12
D-Xylose (Eimer and Amend)	16	8
L-Arabinose	>60	59
Fructose	>60	59
D-Mannose	>60	23
D-Ribose	>60	53
Fructose-6-phosphate	>60	
Fructose-1,6-diphosphate	>60	

Total volume, 2.05 ml.; substrate, 0.2 ml. of a 1 M solution in H_2O; protein, coenzyme, buffer, oxidation-reduction indicator, water, and temperature as in Table V.

TABLE VII

Effect of Glucose Concentration

Final glucose concentration	Oxygen consumption	
	60 min.	120 min.
M	*c.mm.*	*c mm.*
0 050	69	114
0 025	54	88
0.0125	42	68
0.006	26	44
0 003	20	32

Total volume, 5.0 ml.; protein, 2 ml., containing the water-soluble portion from 20 mg. of Preparation B after centrifuging off the insoluble proteins; approximately 13.0 mg. goes into solution; coenzymes, 0.5 ml. each of 50 mg. per cent DPN and TPN in 0.25 M phosphate buffer at pH 7.4; substrate, 0.25 ml. of a 0.0625 M to 1 M solution of D-glucose; buffer, 1.0 ml. of 0.25 M phosphate at pH 7.4; methylene blue, 0.5 ml. of a 1:5000 solution in H_2O; temperature, 38°; center well, 0.25 ml. of 10 per cent NaOH.

or 10 mg. per ml. This was the concentration used to test the effect of varying the substrate concentration. At a 0.050 M final concentration of glucose (Table VII) the oxygen consumption was found to be 114 c.mm. in

2 hours, whereas at 0.003 M final concentration of glucose (6 per cent of 0.050 M) the oxygen consumption was found to be 32 c.mm. in 2 hours. Under comparable conditions and with 0.050 M glucose in the control vessels any substrate which causes uptake in excess of 32 c.mm. in 2 hours would have to be itself oxidized or contain impurities equivalent to at least 6 per cent of D-glucose.

TABLE VIII

Effect of Various Substrates at One Protein Concentration

Experiment	Substrate	Oxygen consumption	
		60 min.	120 min.
		c.mm.	*c.mm.*
1	None	4	10
	D-Glucose	68	126
	D-Galactose (Eastman)	14	25
	D-Galactose (Eimer and Amend)	20	29
	D-Mannose	9	14
	Fructose	10	16
2	None	3	8
	D-Glucose	72	129
	D-Arabinose	34	61
	L-Arabinose	19	30
	D-Xylose (Eastman)	30	53
	D-Lyxose	23	43
3	None	5	10
	D-Glucose	67	123
	D-Glucose-6-phosphate	35	68
	Fructose-6-phosphate	22	36
	Fructose-1,6-diphosphate	25	49
	D-Ribose	12	18

Total volume, 5.0 ml.; substrate, 0 25 ml. of a 1 M solution in H_2O; protein, coenzymes, buffer, methylene blue, and temperature as in Table VII; center well, 0.25 ml. of 10 per cent NaOH.

In Table VIII we see the effect of various substrates on the oxygen consumption at a protein concentration of 20 mg. per vessel. Those substrates which stimulated the oxygen consumption so that it exceeded 32 c.mm. in 2 hours were D-glucose, D-arabinose, D-xylose (Eastman), D-lyxose, D-glucose-6-phosphate, fructose-6-phosphate, and fructose-1,6-diphosphate. These substrates, therefore, are themselves oxidized by Preparation B with added coenzymes or contain impurities equivalent to 6 per cent of glucose.

As a further test of actual oxidation several of the substrates were studied at two protein concentrations (Table IX). The 20 mg. sample per vessel previously used was one concentration and 3 times that amount, or 60 mg.

per vessel, was the other. At the lower protein concentration the substrates which stimulated the oxygen consumption to exceed or nearly to exceed 32 c.mm. in 2 hours were the same as before, namely, D-glucose, D-arabinose, D-xylose (Eastman), D-lyxose, D-glucose-6-phosphate, fructose-6-phosphate, and fructose-1,6-diphosphate. These sugars were all oxidized at a greater rate at the higher protein concentration. D-Galactose (Eastman) and L-arabinose failed to stimulate the oxygen consumption to rise above 32 c.mm. even at the higher protein concentration.

TABLE IX

Effect of Various Substrates at Two Protein Concentrations

Substrate	20 mg. protein		60 mg. protein	
	Oxygen consumption			
	60 min.	120 min.	60 min.	120 min.
	c mm	c mm.	c.mm.	c.mm.
None	5	10	5	8
D-Glucose	72	126	111	195
D-Galactose (Eastman)	8	12	18	26
D-Arabinose	24	44	80	139
L-Arabinose	9	14	11	18
D-Xylose (Eastman)	18	30	35	52
D-Lyxose	22	36	36	56
D-Glucose-6-phosphate	24	44	54	96
Fructose-6-phosphate	20	36	82	161
Fructose-1,6-diphosphate	26	50	82	148

Total volume, 5.0 ml.; protein, 2 ml., containing the water-soluble portion from 20 or 60 mg. of Preparation B after centrifuging off the insoluble proteins; approximately 13.3 and 40 mg., respectively, go into solution; substrate, coenzymes, buffer, methylene blue, and temperature as in Table VIII; center well, 0.25 ml. of 10 per cent NaOH.

It would appear then as though the only five substances which are definitely oxidized by Preparation B in the presence of added methylene blue, DPN, and TPN are D-glucose, D-arabinose, D-glucose-6-phosphate, fructose-6-phosphate, and fructose-1,6-diphosphate. The oxidation of D-xylose (Eastman) and D-lyxose remains uncertain. The small oxidation of all the other sugars appears to be due either to the presence of impurities or to a lack of specificity of the participating enzymes.

The last experiments (Table X) were done with Preparation C, which differs from Preparation B in two respects: special precautions were taken to keep the preparation cold at all stages except during centrifugation when refrigeration was not available, and the lyophilized liver was homogenized in a Waring blendor with 4 volumes of cold distilled water instead of being

ground in a mortar. Further, in preparing the final product for use in the experiments reported below, the protein was dissolved in 0.25 M phosphate buffer of pH 7.4 instead of in water; the protein dissolved completely in the buffer at the concentrations used.

The substrates were studied at two protein concentrations, 20 mg. and 40 mg. per vessel. A substrateless control and a control containing 0.003 M D-glucose in final concentration (6 per cent of 0.050 M D-glucose) were included. All other sugars were used at 0.050 M final concentration of sub-

TABLE X

Oxidation of Various Substrates at Two Protein Concentrations

Final substrate concentration	20 mg. protein		40 mg. protein	
	Oxygen consumption			
	60 min.	120 min.	60 min	120 min.
M	c.mm.	c mm.	c.mm.	c. mm.
No substrate	17	28	20	33
0.050 D-glucose	180	353	388	678
0.003 D-glucose	40	67	61	97
0.050 D-arabinose	45	100	95	166
0.050 D-xylose (Eastman)	70	126	137	229
0.050 D-lyxose	41	79	71	119
0.050 D-glucose-6-phosphate	54	95	88	151
0 050 fructose-6-phosphate	47	80	85	138
0.050 fructose-1,6-diphosphate	49	93	72	138

Total volume, 5.0 ml.; protein, 2 ml., containing 20 or 40 mg. of Preparation C dissolved in 0.25 M phosphate buffer at pH 7.4; the protein is completely soluble; substrate, 0.25 ml. of a 1 M or 0.06 M solution in H_2O to make a final concentration of 0.050 M or 0.003 M, respectively; coenzymes, 0.5 ml. each of 100 mg. per cent of DPN and TPN in H_2O; methylene blue, 0.5 ml. of a 1:5000 solution in H_2O; water, 1.0 ml ; center well, 0.25 ml. of 10 per cent NaOH; temperature, 38°.

strate. D-Glucose, D-arabinose, D-xylose (Eastman), D-lyxose, D-glucose-6-phosphate, fructose-6-phosphate, and fructose-1,6-diphosphate gave values for oxygen consumption in 1 and 2 hours, and at the two protein concentrations that exceeded the 0.003 M D-glucose control. It would appear that all seven of these sugars are oxidized. If they are not oxidized, they should contain impurities equivalent to more than 6 per cent of D-glucose.

DISCUSSION

In 1936 Lipmann (24) and Dickens (25), working independently, formulated a hypothesis to the effect that the aerobic oxidation of glucose monophosphate in yeast might proceed through phosphogluconic acid to 2-ketophosphogluconic acid and hence by decarboxylation to arabinose

monophosphate. Dickens carried the hypothesis further and suggested that by a repetition of the oxidations and the decarboxylations the arabinose phosphate might go to a tetrose phosphate and finally to a triose phosphate. Breusch (8) has more recently made the suggestion that such a scheme, although without phosphorylation, might play a part in mammalian carbohydrate metabolism. He bases his hypothesis on the fact that D-glucose, D-arabinose, glyceraldehyde, and glycoaldehyde, and possibly D-erythrose are all oxidized in the presence of a Latapie mince of cat liver when methylene blue is the oxidation-reduction indicator. It is toward the evaluation of such a scheme as this that the present work is especially directed.

Since Muller (7) had reported that D-galactose and D-xylose were oxidized by preparations of ox liver and since Breusch (8) had found that these two were not oxidized by cat liver, it was necessary to verify the results of one or the other. It appears from our experiments that D-galactose is not oxidized by lamb liver, but that D-xylose is readily oxidized, at least by some preparations. D-Xylose does have in its first 4 carbons the same spatial configuration that exists in the first 4 carbons of D-glucose, which suggests that the oxidation may be due to a lack in specificity of D-glucose dehydrogenase. Muller's (7) results with D-galactose may have been due to the high concentration of substrate that he was using. His final concentrations ranged from 0.125 to 0.50 M, whereas Breusch used 1 mg. to 3 ml. of fluid to make a final concentration of approximately 0.002 M.

Whether the phosphorylated hexoses that are reported oxidized here are directly dehydrogenated is not certain, since the possibility exists that they may be split to trioses, perhaps to phosphoglyceraldehyde, and that the oxygen consumption represents oxidation to phosphoglyceric acid. It is possible also that *Zwischenferment* from red blood cells is present in these preparations, since much blood remains in the liver even though the animals had been exsanguinated.

SUMMARY

1. The preparation of a mixture of partially purified dehydrogenases from lyophilized lamb liver is described.

2. The slow decline in the rate of oxygen consumption by surviving rabbit liver slices is partly prevented by the addition of only fructose-1,6-diphosphate from among a number of phosphorylated hexoses and non-phosphorylated hexoses and pentoses.

3. D-Glucose, D-arabinose, D-xylose, D-lyxose, D-glucose-6-phosphate, fructose-6-phosphate, and fructose-1,6-diphosphate are oxidized in the presence, but not in the absence, of methylene blue when diphosphopyridine nucleotide, triphosphopyridine nucleotide, and a partially purified protein

preparation from lyophilized lamb liver are added.   It is not suggested that the first step in the oxidation of all seven of these compounds is dehydrogenation.   D-Galactose and several other sugars are not oxidized.

4. A modification in the first step in the synthesis of D-glucose-6-phosphate, *i.e.* in the synthesis of diacetone glucose, is presented.

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THE EFFECT OF ADRENAL CORTEX AND ANTERIOR PITUITARY EXTRACTS AND INSULIN ON THE HEXOKINASE REACTION*

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It was stated in preliminary reports (1, 2) that the hexokinase reaction, glucose + adenosine triphosphate → glucose-6-phosphate + adenosine diphosphate, in muscle extracts from rats made diabetic with alloxan was markedly inhibited by the addition of adrenal cortex extract, while no inhibition was observed when adrenal cortex was added to extracts of normal rat muscle. Hexokinase preparations (muscle, brain) of normal animals could be inhibited, however, by the addition of certain protein fractions of the anterior pituitary, particularly in the presence of adrenal cortex extract, and this inhibition, like that observed in diabetic extracts, could be counteracted by insulin.

Other experiments indicated that insulin was not an accelerator of the reaction, since it did not exert an effect unless the system was inhibited by adrenal cortex alone in diabetic extracts and by pituitary plus adrenal cortex in normal extracts. This raised the question whether the diabetic extracts contained an inhibitory factor similar to that prepared from the anterior pituitary. On the assumption that the phenomena are related, the experiments with extracts of diabetic rat muscle and the preparation of inhibitory pituitary fractions will be described in this paper.

EXPERIMENTAL

Hexokinase in Muscle Extracts of Diabetic Rats—In some of the early experiments rats were injected intraperitoneally with 200 mg. of alloxan per kilo; the degree of glycosuria varied considerably in the different animals and there were a number of fatalities. More uniform results were obtained when the procedure of Kass and Waisbren (3) was adopted, which consists in fasting the animals for 48 hours before injecting the above dose of alloxan subcutaneously. After the injection the animals were given food *ad libitum* and the urine was collected in individual metabolism cages. The animals showing marked glycosuria were killed about 2 days after the injection. About 10 gm. of leg muscle were rapidly excised and minced

with scissors on a glass plate in a cold room at 5° to a medium degree of fineness. The minced muscle was rubbed vigorously for 4 minutes in a mortar after addition of 1.5 volumes of water. In a few experiments a solution of 0.05 M phosphate (pH 7.5)-0.08 M NaCl was used for extraction, which did not seem to make any difference in the results. The muscle pulp was either centrifuged or strained through gauze, and the extract tested immediately. The pH of the water extract was about 6.4 and of the phosphate-saline extract 6.9.

The extraction must accomplish two things; it must yield an active hexokinase preparation and it must at the same time give a maximum yield of the inhibitory factor. The optimal conditions for dealing with these two variables have not been worked out. What limits the possibilities is the great instability of the inhibitory factor, making speed of the preparation of the extract and maintenance of a low temperature prime prerequisites of a successful experiment.

The muscle extract (0.8 cc.) was added to the main compartment of two Warburg manometer vessels, each of which contained 1 cc. of 0.02 M $MgCl_2$, 0.06 M $NaHCO_3$, 0.1 cc. of the Upjohn adrenal cortex extract, and in one case 0.1 cc. of H_2O and in the other 0.1 cc. of insulin[1] solution (100 γ). The side bulb of the Warburg vessels contained in each case 0.1 cc. of 1 per cent glucose, 0.15 cc. of 0.9 M NaF,[2] 0.05 M $NaHCO_3$, and 0.1 cc. of 0.1 M adenosine triphosphate. The contents of the two Warburg vessels (2.35 cc.) differed therefore in only one respect; namely, that insulin was present in one and not in the other.

The vessels were gassed for 4 minutes with 5 per cent CO_2-95 per cent N_2 and equilibrated for 5 minutes at 30° (pH about 7.5). The contents of the side bulbs were mixed with the main compartments and liberation of CO_2 was measured for 10 to 15 minutes or longer, depending on the activity of the extracts. At the end of the incubation period, aliquots of the vessel contents were precipitated with 1 volume of $Ba(OH)_2$ and 1 volume of $ZnSO_4$ according to Somogyi, and the glucose determined in the filtrate according to Nelson (4). The $Ba(OH)_2$ and $ZnSO_4$ solutions should be so adjusted that the filtrates (pH of filtrate about 7.2) are free of either Ba or Zn ions. A third sample was fixed before incubation in order to determine the initial glucose content. About 1 mg. of glucose was present initially and, since the experiments were so arranged that a substantial part of the glucose disappeared, the calculations are based on large differences in the glucose readings and involve a multiplication factor of only 12. Control experiments showed that added hexose phosphate esters were precipitated

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[1] An amorphous preparation of insulin, assaying 20 units per mg., was kindly supplied by Dr. Chen of Eli Lilly and Company.

[2] NaF was added in order to inhibit adenylpyrophosphatase.

by the Ba-Zn treatment and that the deproteinized filtrate contained only a negligible amount of non-sugar reducing substances.

Because of their greater accuracy the glucose determinations were relied upon to calculate the per cent inhibition of glucose utilization. For this reason the values for CO_2 evolution are not reported in detail, except to illustrate (Fig. 1) that an inhibition, when present, can be detected by the manometric method and that addition of adrenal cortex extract increases the inhibition very markedly.[3] In most cases there was a rough quantitative agreement between the CO_2 readings and the subsequent glucose determinations. The manometric procedure was used because it provided for continuous mixing, a factor which was found to be of importance; it also indicated a suitable time at which to interrupt the experiment for glucose determinations and it served as an independent method for evaluating the experiment. All values in Tables I to VII are calculated for an incubation period of 10 minutes; as shown in Fig. 1, the reaction rate is approximately linear.

In Table I are summarized experiments on muscle extracts of nine diabetic rats and one diabetic rabbit. Inhibitions ranging from 27 to 64 per cent were observed when the extracts were tested immediately after preparation. Also included are four experiments on extracts of normal rat muscle; they show that insulin had no stimulatory effect on the hexokinase reaction and they illustrate the precision of the measurements when no inhibition is present. Other control experiments belonging to this series which are not specifically recorded consisted in testing the activity of muscle extracts of normal rats without any additions and with the addition of insulin or adrenal cortex separately or combined; in all of these cases the disappearance of glucose did not vary by more than ±5 per cent.

That the effect of insulin consists in the removal of an inhibition of hexokinase activity and not in an acceleration of the reaction is also shown in the last three columns of Table I. When the muscle extracts were kept for 45 to 270 minutes at 0° and then retested, the activity of the sample without insulin had risen in most cases to the level observed in the

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[3] In these extracts there is a considerable production of CO_2 without the addition of glucose (Fig. 1). This is due, in part, to the presence of some glucose in extracts of diabetic rats. One cannot therefore deduct the "basal" values from those obtained in the presence of glucose. Furthermore, NaF does not completely suppress adenosinetriphosphatase activity, and there is also some acid production in samples to which no adenosine triphosphate has been added. Two equivalents of CO_2 are evolved for each molecule of glucose used, because both the glucose added and the hexose monophosphate formed in the hexokinase reaction serve as phosphate acceptors. The inhibition concerns only the first reaction. All these factors decrease the accuracy of the manometric determination and it serves merely to give the degree of inhibition of the hexokinase reaction.

presence of insulin. In only two experiments did the inhibition persist after the extract had been stored for an hour or longer at 0°. In two experiments the extracts were frozen at $-20°$ immediately after preparation; on thawing and retesting, the hexokinase was no longer inhibited.[4] These experiments show that the inhibitory factor is very labile, and, as will be
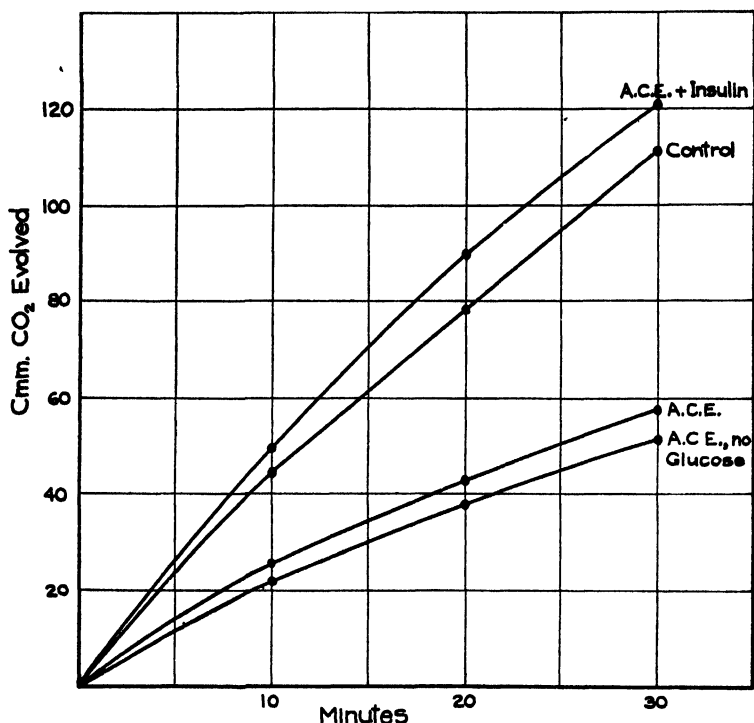
FIG. 1. Effect of adrenal cortex extract (ACE) and insulin on CO_2 production in extract of muscle from rats made diabetic with alloxan. The composition of the reaction mixture is given in the text. The control sample contained neither ACE nor insulin. Terminal glucose determinations showed that the inhibition was 67 per cent for the sample which contained ACE, as compared to the sample which contained ACE plus insulin.

shown later, it resembles in this respect the inhibitory factor prepared from the anterior pituitary. Whether or not the disappearance of the inhibitory factor is due to enzymatic destruction has not been determined with certainty.

A total of thirty experiments on diabetic and six experiments on normal

[4] In a few instances the inhibitory action was not destroyed by freezing the muscle extract.

rat muscle was carried out; these are summarized in Table II. None of the normal extracts showed significant inhibitions. Of the diabetic ex-

TABLE I

Effect of Insulin on Glucose Utilization in Extracts of Muscles from Rats Made Diabetic with Alloxan

The composition of the reaction mixture is given in the text. Incubated for 10 minutes at 30°; adrenal cortex extract was added to all the samples.

After injection of alloxan	Glucose utilized per 2 35 cc. reaction mixture		Inhibition without insulin	Extract stored at 0° before retesting	Glucose utilized when retested		Inhibition without insulin
	No insulin	Insulin			No insulin	Insulin	
hrs	γ	γ	*per cent*	*min*	γ	γ	*per cent*
41	215	575	64	70	560	550	0
48	175	380	54	Frozen	260	260	0
47	113	238	53	120	128	189	32
43	144	264	45	75	160	242	34
27	460	825	44	60	795	810	2
41	128	218	41	270	188	198	5
43	535	865	38	105	795	830	4
50	360	580	38	Frozen	405	405	0
43*	170	255	33	60	165	160	−3
48	415	570	27	45	510	490	0
Normal	166	186	11				
"	336	346	3				
"	378	376	0				
"	210	203	0				

* Diabetic rabbit.

TABLE II

Summary of Data on Inhibition of Hexokinase Reaction in Muscle Extracts of Diabetic Rats

Per cent inhibition calculated from glucose measurements with and without insulin.

No of rats	Per cent inhibition		Remarks
	Range	Average	
7	42–76	51	Injected with alloxan
8	21–38	34	" " "
15	0–13	3	" " "
6	0–11	3	Normal controls

tracts fifteen showed inhibitions ranging from 76 to 21 per cent, while fifteen showed either inhibitions of less than 14 per cent, which were not regarded as significant, or no inhibitions at all. Although the diabetes

varied in intensity in the different animals, the most probable explanation
for the negative results is the instability of the inhibitory factor. Since a
short period of storage at 0° leads to its disappearance (Table I), it seems
likely that this process was going on continuously during the preparation of
the extract and that in some cases the inhibition had disappeared com-
pletely before the enzyme test was started.

Graded effects of insulin and adrenal cortex extract on the hexokinase
reaction in diabetic muscle extract are shown in Fig. 2. When 0.05 cc. of
the Upjohn extract was added to 2.35 cc. of the reaction mixture, it pro-
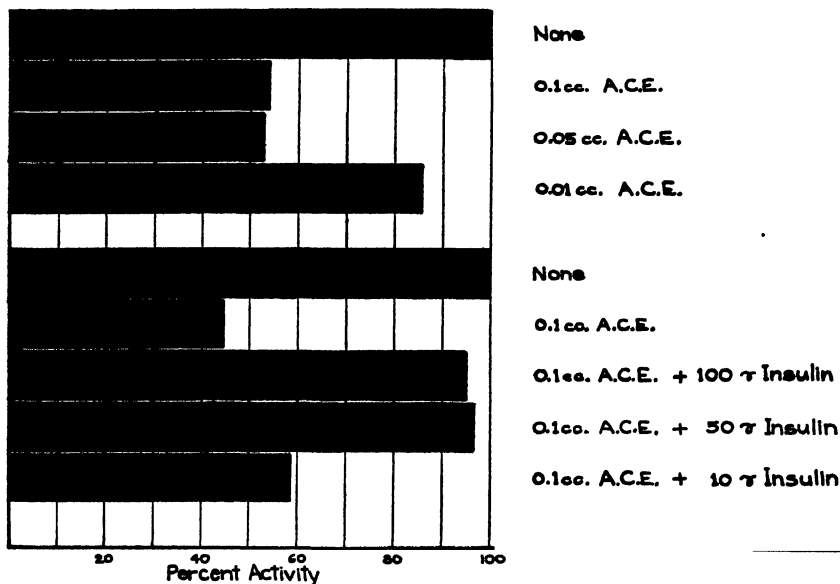
Fig. 2. Effect of Upjohn adrenal cortex extract (ACE) and of insulin on hexo-
kinase activity of muscle extracts of rats made diabetic with alloxan. Additions
per 2.35 cc. of reaction mixture.

duced as much inhibition as 0.1 cc., while 0.01 cc. was less effective. Both
100 and 50 γ of insulin counteracted the inhibition in the presence of 0.1
cc. of adrenal cortex extract, while 10 γ gave only a partial reversal of the
inhibition.

Preparation of Brain Hexokinase—Fresh beef brain was chilled on ice,
and, after removing the adhering membranes, the gray matter was stripped
off and homogenized in a Waring blendor in the cold. The homogenate
was slowly added to 10 volumes of acetone (−5°) while the blendor was in
operation and was homogenized for 2 minutes. The material was filtered
on a Büchner funnel and the filter cake washed with cold acetone and dried

in vacuo over H_2SO_4. The dry powdered material could be stored in a desiccator in the cold for several months without loss of activity. Extracts were prepared from the powder with 8 volumes of ice-cold distilled water. After centrifugation (10,000 R.P.M.), the clear supernatant fluid was used as a source of hexokinase. These extracts kept their activity fairly well for several hours when stored at 0° and could also be stored frozen for a week or longer without significant loss of activity.

Fractionation of the aqueous extract of the acetone powder with ammonium sulfate between 0.3 and 0.5 saturation doubled the activity per mg. of protein, and further purification could be effected by precipitation between 0.45 and 0.6 saturation. This partially purified hexokinase did not convert added glucose-1-phosphate to glucose-6-phosphate or to glucose and inorganic phosphate during 20 minutes of incubation at 30° in the presence of 0.01 M $MgCl_2$ and 0.08 M NaF. The same enzyme preparation formed large amounts of hexose-6-phosphate and hexose diphosphate from glucose and adenosine triphosphate. This indicates that, as in the case of yeast hexokinase (5), the primary phosphorylation product of brain hexokinase is glucose-6-phosphate.

Preparation of Inhibitory Factor from Anterior Pituitary—The concentration of active material in the crude extracts described below was found to be too low to demonstrate any effect when 0.4 cc. was added to the hexokinase test system.[5]

A number of procedures were tried for concentrating the active material, of which four will be described. In all cases the pituitary glands were obtained at the slaughter-house as rapidly as possible after the death of the animal and frozen in solid CO_2. In most cases glands which had been stored frozen for not more than 3 days were used, since longer storage sharply reduced the number of successful preparations.

In the case of beef glands, the anterior lobes were separated from the posterior lobes and connective tissue while still in the frozen state. In the case of sheep the whole glands were used. The frozen tissue was first passed through a meat grinder and then ground in a mortar with sand. This and all further steps were carried out in a cold room at 5°. To the tissue paste were added 5 volumes of distilled water. Further steps are described below. All centrifugations were carried out at 10,000 R.P.M. by means of the multispeed attachment of the International centrifuge.

[5] When 3 to 5 cc. of the crude extract were injected intraperitoneally in normal rats and a muscle extract was prepared 2 to 3 hours later, the hexokinase activity in these muscle extracts showed in a number of cases the same behavior as in extracts of diabetic rat muscle. There was an inhibition of hexokinase activity in the presence of adrenal cortex extract, when compared to a sample to which adrenal cortex extract and insulin had been added. The percentage of positive results was smaller than in the series with diabetic rats.

The crude extract was centrifuged for 10 minutes, while all further centrifugations were for only 2 to 5 minutes. The pH was controlled by means of a glass electrode.

Procedure 1—To the ground glands suspended in water was added 0.2 N Ba(OH)$_2$ to bring the pH to 9.8 to 10.2. After 30 minutes of extraction the material was centrifuged. The turbid supernatant fluid was brought to pH 6.8 with 0.05 N H$_2$SO$_4$ and the precipitate discarded after centrifugation. More H$_2$SO$_4$ was added to the clear supernatant fluid until pH 5.7 was reached. The precipitate was collected by centrifugation, washed with distilled water on the centrifuge, and dissolved in the smallest possible volume of 0.05 M Na$_2$HPO$_4$. The solution was diluted ten times with water, the pH adjusted to 6.8, and centrifuged. From the supernatant fluid two protein fractions were obtained, one precipitating between pH 6.8 and 6.3 and the other between 6.3 and 5.7. Both were dissolved in 0.05 M Na$_2$HPO$_4$ and immediately tested for inhibitory action. The combined fractions represented about 20 per cent of the total extracted proteins.

Procedure 2—The tissue was extracted with Ba(OH)$_2$ or NaOH for 30 minutes at pH 10 to 11, as described in Procedure 1. After centrifugation the alkaline supernatant fluid was treated with 0.1 volume of an aluminum hydroxide, Cγ, solution containing 20 mg. of Al(OH)$_3$ per cc. The adsorbed material was centrifuged off, washed with water, and eluted with 0.1 M Na$_2$HPO$_4$. The clear eluate was used for the test, it contained about 4 per cent of the extracted protein.

Procedure 3—0.05 N H$_2$SO$_4$ was added to the crude aqueous suspension of the glands until the pH was 5.7, and the suspension was centrifuged immediately. With this procedure only one-half as much protein is extracted as with Procedures 1 and 2. The supernatant fluid was brought to pH 5.3 with more H$_2$SO$_4$ and 2 volumes of distilled water were added. The precipitate which formed was centrifuged off, dissolved in 0.05 M Na$_2$HPO$_4$, and tested immediately. About 14 per cent of the extracted protein was recovered in this fraction.

Procedure 4—The undiluted supernatant fluid, pH 5.3, described in Procedure 3, was treated with 0.1 volume of Al(OH)$_3$. The adsorbed material was washed with water and eluted with 0.1 M Na$_2$HPO$_4$. The yield was about 15 per cent of the extracted protein.

The test system, total volume 2.35 cc., had the same final composition as that described for muscle extract, except that 0.3 cc. of brain hexokinase was used as the source of the enzyme and 0.4 cc. of pituitary fraction containing 0.02 M NaHCO$_3$ was placed in a second side bulb of the Warburg vessels. After gassing with 5 per cent CO$_2$-95 per cent N$_2$ and temperature equilibration, the contents of the main compartment and side bulbs were mixed to start the reaction. As in the case of the experi-

ments with muscle extracts, two identical vessels were prepared, except that one contained 100 γ of insulin while none was added to the other. Table III shows that treatment of insulin with 0.05 N KOH for 3 hours at 37° destroys its activity in the hexokinase test system.

TABLE III

Effect of Treatment of Insulin with Alkali on Its Ability to Release Brain Hexokinase from Pituitary Inhibition

Amorphous insulin incubated in 0.05 N KOH for 3 hours at 37°. 1.7 mg. injected intravenously into each of two rabbits caused no lowering of blood sugar levels. Pituitary fractions prepared according to Procedure 1. Incubated for 10 minutes at 30° without insulin and with 100 γ of native or alkali-treated insulin. Adrenal cortex extract was added to all the samples

Pituitary fraction	Pituitary protein added	Glucose utilized per 2.35 cc reaction mixture			Inhibition	
		No insulin	Alkali-treated insulin	Native insulin	No insulin	Alkali-treated insulin
	mg	γ	γ	γ	*per cent*	*per cent*
pH 6 2–5 7	10 9	270	300	455	41	34
" 6 8–6.2	4 2	385	375	445	13	16

TABLE IV

Effect of Pituitary Extract and Adrenal Cortex Extract on Brain Hexokinase in Absence and Presence of Insulin

The pituitary preparations (APE) were made according to Procedure 1 (pH 6 3 to 5 7 precipitates) 0 1 cc of the Upjohn adrenal cortex extract (ACE) and 100 γ of insulin (I) were added   Incubated for 10 minutes at 30°.

Experiment No	Glucose utilized per 2 35 cc reaction mixture				Inhibition (calculated from 4th and 5th columns)
	No additions	APE	APE + ACE	APE + ACE + I	
	γ	γ	γ	γ	*per cent*
1	552	510	355	530	33
	556	535	400	525	24
2	532	527	310	518	40
3	342	225	198	348	43

Table IV shows three experiments with a pituitary fraction prepared by Procedure 1; in two of these experiments there was no inhibitory effect unless adrenal cortex extract was also added; in the third experiment the pituitary fraction alone had an inhibitory effect, which was increased by the addition of adrenal cortex extract. For this reason adrenal cortex extract was added in all assays for pituitary inhibitor. Table IV also shows the activity of the brain hexokinase without any additions and

illustrates the fact that the inhibition is removed by insulin. Control experiments have also been carried out which show that adrenal cortex extract in the absence of the pituitary inhibitor had no effect on the hexokinase reaction.

TABLE V

Effect of Pituitary Extracts on Beef Brain Hexokinase

Adrenal cortex extract was added to all the samples; incubated for 10 minutes at 30°.

Procedure No.	Species	Pituitary protein added	Glucose utilized per 2.35 cc. reaction mixture		Inhibition without insulin	Extract stored at 0° before retesting	Glucose utilized when retested		Inhibition without insulin
			No insulin	Insulin			No insulin	Insulin	
		mg.	γ	γ	*per cent*	*min.*	γ	γ	*per cent*
1	Beef		135	230	41				
	"		290	450	36				
	"	6.2	320	435	26				
	Calf	7.7	325	490	34				
	Sheep		300	500	40				
	"	4.7	145	230	37				
2	"	3 1	130	360	64				
	"	1.0	245	360	32	70	280	305	8
	"		185	265	30	120	260	260	0
	"	3 0	195	275	29				
	"	2 8	275	385	29				
3	Horse*		230	440	48	60	485		
	" *		330	485	32	120	370	350	−5
	Sheep	5 2	120	520	77				
	"		145	340	57	70	360	400	10
	"	2.8	255	495	49				
	"		280	480	42	Frozen	525	505	−4
4	"	5 4	105	445	76	50	385		
	"	5 1	220	390	44				
	"		155	275	44	30	305	320	5
	"		275	350	21				

* Lyophilized glands.

Representative experiments with fractions obtained by the four procedures are shown in Table V, while a summary of all experiments on twenty-seven different batches of glands is given in Table VI. None of the procedures invariably yielded an active extract. In Table VI all experiments on glands stored not longer than 3 days are listed as negative, if the inhibition was less than 14 per cent. Although all positive and negative experiments are recorded, it should be understood that the actual number of each is influenced by the number of trials that were made with each batch of glands.

Actually each of the twenty-seven batches yielded an active extract by one or the other method of preparation.

There were twelve other positive experiments in which other procedures were tried, such as fractionation of the alkaline extract with ammonium sulfate between 0.2 and 0.45 saturation, dialysis and isoelectric precipitation between pH 6.8 and 5.8, adsorption with $Al(OH)_3$ at different pH levels, and the use of muscle extract instead of brain hexokinase as the test system. In still other experiments the measurement of CO_2 production was omitted and the reaction mixture was incubated in test-tubes without shaking. Although this method appeared satisfactory, there were several tests in which the sample incubated in Warburg vessels gave a positive result, while the same pituitary fraction, when incubated in test-tubes without shaking, showed no inhibitory action.

TABLE VI

Summary of Inhibitions of Hexokinase Activity Obtained with Fractions of Pituitary Gland

Procedure No.	No. of preparations		Per cent inhibition of positives		Remarks
	Positive	Negative	Range	Average	
1. pH 6 8–6 3 ppt	7	4	14–36	25	7 batches (4 beef, 3 sheep)
1 " 6.3–5 7 "	6	4	29–41	34	
2	6	3	14–64	33	5 batches (sheep)
3	17	7	15–77	41	9 " "
4	6	5	20–76	46	6 " "

In Procedure 1, Table VI, the pH 6.8 to 6.3 precipitate was less active per mg. of protein than the pH 6 3 to 5.7 precipitate. Acid extraction followed by isoelectric precipitation between pH 5.7 and 5.3 or adsorption of the acid extract with $Al(OH)_3$ (Procedures 3 and 4, Table V) yielded more active extracts, since 3 to 5 mg. of protein per test gave inhibitions of 44 to 77 per cent. Only 1 to 3 mg. of protein were required for 29 to 64 per cent inhibition when Procedure 2 was used. Active preparations were also obtained on three occasions from a lyophilized horse pituitary powder kindly supplied by Dr. Campbell of Eli Lilly and Company. After more prolonged storage this powder failed to yield active material. When the purified fractions of pituitary were lyophilized, the activity was lost.

In several cases the tests were repeated after the pituitary fractions had stood for 30 to 120 minutes in an ice bath or after they had been frozen, but invariably the inhibitory action had disappeared. As shown in Table V, in these cases the hexokinase activity in the sample with insulin remained

about the same, while the sample without insulin showed in each case a greater activity than in the test with the freshly prepared pituitary extract. These observations duplicate those recorded in Table I with muscle extracts of diabetic rats. Various attempts were made to increase the stability of the inhibitory fractions by keeping them at various hydrogen ion concentrations or in an atmosphere of N_2, or by adding various ions or a boiled juice of pituitary glands, but so far none of these and other procedures have been successful.

During a visit of Dr. Li an opportunity was afforded for testing three highly purified pituitary hormones prepared by him in Dr. Evans' laboratory. Growth, adrenotropic, and lactogenic hormone preparations had no inhibitory effect on the hexokinase reaction (Table VII).

TABLE VII

Absence of Inhibitory Effect of Highly Purified Pituitary Hormones

1 mg of each was added to 2 35 cc. of reaction mixture. Adrenal cortex extract was added to all the samples; incubated for 10 minutes at 30°.

Hormone	Glucose utilized per 2 35 cc. reaction mixture		Inhibition
	No insulin	Insulin	
	γ	γ	*per cent*
Growth	340	340	0
Adrenotropic	380	410	7
Lactogenic ..	300	330	9

DISCUSSION

Since adrenal cortex extract produces a marked inhibition of hexokinase activity in muscle extracts of diabetic rats, but has no effect on the hexokinase activity of normal muscle or brain extracts unless a pituitary fraction is also added, the authors would like to draw the tentative conclusion that there is present in muscle extracts' of diabetic rats an inhibitory substance, possibly of pituitary origin. Such a substance may also be present in extracts of normal muscle, but it cannot be demonstrated, either because less is present or because there is enough insulin in the extracts to nullify its action.

Some support for the above concept is derived from the fact that adrenal cortex extract fails to inhibit diabetic muscle preparations which have been stored for short periods at 0°. This certainly indicates the loss of a factor participating in the inhibition, and its remarkable lability resembles that observed with the inhibitory substance prepared from pituitary glands. Furthermore, the response of diabetic muscle extracts to increasing amounts

of adrenal cortex extract is of the type to be expected if another substance were participating in the inhibition and were the limiting factor determining the maximum degree of inhibition obtainable with an excess of adrenal cortex extract. The final proof for the pituitary origin of this substance will rest on the demonstration that adrenal cortex extract fails to inhibit the hexokinase activity of muscle extracts from hypophysectomized rats treated with alloxan.

Whatever the nature of the inhibition of the hexokinase reaction, there can be no doubt that insulin exerts an antagonistic effect and that this represents a reproducible *in vitro* effect of insulin. In every case in which an inhibition was obtained, addition of insulin completely abolished the inhibitory effect. The possibility that this might be a non-specific protein effect is made extremely unlikely by the high concentration of other proteins in the test system and by the abolition of the effectiveness of insulin by mild treatment with alkali.

The difficulty in obtaining consistent inhibitions of the hexokinase reaction is due largely to the lability of the pituitary factor. In a crude extract of the pituitary, the factor is relatively stable over a wide range of pH, since active material may be obtained after extraction at pH 10 for periods up to 2 hours, or after extraction at pH 5.7 for somewhat shorter periods, while the partially purified material loses its activity completely during even shorter periods of standing at either pH. It cannot be decided at present whether the rapid loss in potency is the result of an enzymatic destruction of the active substance or the result of some physicochemical change such as denaturation, oxidation, or formation of an inactive complex.

Efforts are also being directed toward elucidating the nature of the active substance in adrenal cortex extract. Preliminary experiments indicate that the substance is present in the so called "amorphous fraction" (2).

The possible physiological significance of the observations recorded in this paper has been discussed elsewhere (6) and it has been pointed out that they are in agreement with Houssay's and Long's experiments, which show that the anterior pituitary and the adrenal cortex exert an inhibitory effect on the utilization of blood sugar in depancreatized animals. It has also been emphasized that insulin may have other points of action besides its effect on the hexokinase reaction.

SUMMARY

1. The hexokinase activity of extracts of muscle of diabetic rats can be inhibited by the addition of adrenal cortex extract. Out of thirty different muscle extracts from rats made diabetic by injection of alloxan, fifteen showed inhibitions ranging from 21 to 76 per cent, while fifteen showed

inhibitions of 14 per cent or less. Maximum inhibition is obtained with 0.05 cc. of adrenal cortex extract (Upjohn) per 2.35 cc. of reaction mixture, larger amounts having no further effect.

2. The hexokinase activity of extracts of normal rat muscle or beef brain is not inhibited by adrenal cortex extract, but can be inhibited 30 to 75 per cent by the further addition of 1 to 7 mg. of a protein fraction from the anterior pituitary.

3. Either of the above inhibited systems serves as a suitable test object for the demonstration of a reproducible *in vitro* effect of insulin on the hexokinase reaction, consisting in the complete removal of the inhibition. About 50 γ of insulin are sufficient to counteract the effect of 0.1 cc. of adrenal cortex extract on diabetic muscle extracts. Treatment of insulin with 0.05 N KOH for 3 hours at 37° abolishes its effectiveness in the hexokinase test system.

4. It is proposed that the inhibitory effect of adrenal cortex extract alone on diabetic muscle extracts depends on the presence in the latter of an inhibitory factor, presumably of pituitary origin.

5. Various procedures for the preparation of the inhibitory factor from pituitary glands are described. Acid or alkaline extractions may be used, followed by either isoelectric precipitation or adsorption on alumina and elution of the active material with phosphate. All preparations obtained so far are exceedingly unstable, and in this respect bear a striking resemblance to the inhibitory substance in diabetic muscle extracts.

6. Highly purified preparations of the adrenotropic, lactogenic, and growth hormones have been tested for inhibitory action with negative results.

7. Beef brain hexokinase has been partially purified by ammonium sulfate fractionation, and it has been shown that the hexokinase reaction in brain, just as in yeast, consists in a phosphorylation of glucose by adenosine triphosphate in the 6 position without the intermediate formation of glucose-1-phosphate.

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THE DISTRIBUTION, RETENTION, AND EXCRETION OF RADIOPHOSPHORUS* FOLLOWING THYROPARATHYROID-ECTOMY, OR BILATERAL NEPHRECTOMY, AND THE ADMINISTRATION OF PARATHYROID EXTRACT†

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In 1898, ver Eecke (1) reported a conspicuous decrease in the excretion of phosphorus in the urine after thyroidectomy, and attributed it to the loss of thyroid function. In 1911, Greenwald (2) found that the diminished urinary excretion of phosphorus following thyroidectomy was due to the concurrent parathyroidectomy, and not to the removal of the thyroid. Greenwald (3) observed that following parathyroidectomy in dogs there occurred a marked retention of acid-soluble phosphorus in the blood and a diminished excretion of phosphorus in the urine. This observation was subsequently confirmed by many investigators.

In later studies, Greenwald (4) found that the amount of phosphorus in the urine of fed dogs during the first 24 hours following parathyroidectomy might be reduced to as low as 2 per cent of the preoperative value. Furthermore, he observed that the very marked retention of phosphorus in the first few days after parathyroidectomy was followed after the appearance of tetany by an increased urinary excretion of phosphorus. From his experimental results, Greenwald (4) reasoned that the failure of parathyroid function must occur immediately upon removal of the glands.

By the tracer technique, it has been observed in our experiments and in a preceding investigation (5) that the retention of labeled phosphate entering the tissues is affected differently in experimental hyperparathyroidism than in experimental hypoparathyroidism. In the former condition there was an increased retention of the administered radiophosphorus in the liver and the kidneys, and an accelerated urinary excretion of radiophosphorus that reduced the amount taken up by the bone. In the latter condition, the marked retention of the administered radiophosphorus in the bone and the other tissues is attributed to a greatly diminished urinary

excretion of radiophosphorus, arising from the sudden withdrawal of parathyroid hormone.

The data presented in this paper support the view that the parathyroid hormone influences phosphorus metabolism by acting directly upon the kidneys.

EXPERIMENTAL

Young adult rats of the Sprague-Dawley strain were used. They were reared on a diet consisting of Purina fox chow, supplemented by greens once weekly. The paired animals, shown in Tables I to V, were litter mates of the same sex. The experimental animals were subjected to thyroparathyroidectomy or bilateral nephrectomy while under ether anesthesia.

The tracer dose of phosphate used in the experiments consisted of 0.5 ml. of an aqueous solution of 3 mg. of phosphorus in the form of Na_2HPO_4, containing 8 to 15 microcuries. The labeled phosphate was administered intraperitoneally, and parathyroid extract,[1] when also employed, was injected subcutaneously. Immediately after the injection of the labeled phosphate, the experimental animal and its control were placed in separate wire bottom cages over urine-feces separators (6).

At the end of the desired period, measured from the time of the administration of the labeled phosphate, blood was collected from the cut tail of the unanesthetized animal, or in some instances from the severed carotid arteries. The blood sample was used for the determination of serum calcium and serum inorganic phosphorus, or for the measurement of radioactivity. Serum calcium was determined by a modification (7) of the Kramer-Tisdall method (8), and serum inorganic phosphorus was determined by Holman's method (9).

The animals were killed by a blow on the head, or by decapitation, and the desired tissues removed for the measurement of radioactivity. The preparation of the tissue samples and the excreta for the measurement of activity, and the method used for their measurement are described in a previous publication (5). A further description of the five series of experiments follows.

Series I—The food intake of the first four pairs of animals (Table I) was not restricted either before or after the experimental animals were subjected to thyroparathyroidectomy. The other experimental animals and their controls (Table I) consumed the stock diet *ad libitum* up to the time of the operation. After the operation, the controls were limited to about the same amount of food consumed by the animals operated on. At various intervals, ranging from a few hours to approximately 25 days

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[1] Manufactured by Eli Lilly and Company.

after the operation, the experimental animals and their controls were injected with labeled phosphate. 18 hours later the animals were sacrificed. The serum calcium values and the activity measurements of the tissues and excreta are shown in Table I.

Series II—The animals used in these experiments received the stock diet *ad libitum* up to and including the experimental period. Approximately 24 hours after thyroparathyroidectomy, each experimental animal was injected with a single dose of parathyroid extract, ranging from 500 to 20 units in potency. 1 hour later a tracer dose of phosphate was administered to each experimental animal and to its normal control. After an interval of 18 hours, the animals were killed. The serum calcium and serum inorganic phosphorus values and the activity measurements of the tissues and excreta are shown in Table II.

Series III—The animals of Group 1 (Table III) were allowed the stock diet *ad libitum* up to and including the experimental period. The animals of Group 2 were restricted to 10 gm. of the stock diet, daily, for the 3 days just preceding the experimental period, and during the experimental period they had access to water only. Approximately 24 hours after thyroparathyroidectomy, each experimental animal of Groups 1 and 2 was injected with 10 units of parathyroid extract. 1 hour later a tracer dose of phosphate was administered to each experimental animal and to its thyroparathyroidectomized control. After an interval of 18 hours, the animals were sacrificed. The serum calcium and serum inorganic phosphorus values and the activity measurements of the femurs and the urine are shown in Table III.

Series IV—The animals used in these experiments were permitted 10 gm. of the stock diet, daily, for the 3 days just preceding the experiment in which they were used. During the experimental period they had access to water only. 2 or 3 hours after thyroparathyroidectomy, the experimental animals were injected with 2.5 or 5 units of parathyroid extract. 20 minutes after the injection of the parathyroid extract, a tracer dose of phosphate was administered to each experimental animal and to its respective thyroparathyroidectomized control. At the end of the various experimental periods (Table IV) the rats were decapitated while being held over the metabolism cage. The urine retained in the bladder after death was added to that recovered by washing the cage and the urine-feces separator.

Eighteen normal rats were subjected to the same dietary restriction as that imposed upon the animals operated on. Nine of these animals were each injected with 5 units of parathyroid extract. 20 minutes later each experimental animal and its control received an injection of labeled phos-

TABLE I

Effect of Thyroparathyroidectomy upon Serum Calcium, and upon Distribution, Retention, and Excretion of Radiophosphorus

The experimental and control animals are designated by the letters E. and C., respectively.

No. of pairs	Weight of animal		Time after operation	Serum calcium		Liver		Skeletal muscle		Femurs		Kidneys		Stomach and small intestine		Large intestine		Feces and contents of gastrointestinal tract		Urine	
						Per cent* of administered radiophosphorus per gm. tissue								Per cent* of administered radiophosphorus recovered							
	E.	C.		E.	C.	E.	C.	E.	C.	E.	C.	E.	C.	E.	C.	E.	C.	E.	C.	E.	C.
	gm.	gm.	days	mg. per cent	mg. per cent																
4	187	186	1	7.84	10.60	0.96	0.72	0.26	0 20	2 44	1.75	0.71	0 52	3.74	3.48	0.62	0.57	2.64	3.65	4.94	17.96
	±29	±26		±1 09	±0.52	±0.02	±0 14	±0.03	±0.03	±0.94	±0.57	±0 09	±0.03	±0.43	±0.41	±0.11	±0.16	±0.27	±0.26	±0.88	±4.99
3	168	172	2–5	7 25	9 56	1 26	0 96	0 24	0.27	2.42	2.02	0.92	0.68	3.59	3.39	0.71	0.66	5.28	3.60	14.18	18.94
	±22	±21		±0 88	±0.29	±0 25	±0.11	±0.04	±0.05	±0 70	±0.12	±0 25	±0.08	±0.52	±0.14	±0.08	±0.08	±0.09	±0.96	±4.40	±5.80
3	216	223	7–12	7.37	10.70	0 83	0 61	0.12	0.17	2 23	1 99	0 64	0 55	3.84	4.08	0.75	0.70	3.86	3.95	12.27	15.56
	±20	±29		±1.17	±0.13	±0 05	±0 11	±0 02	±0.02	±0.72	±0.53	±0 08	±0 08	±0.40	±0.20	±0.18	±0.02	±0.21	±0.82	±1.48	±2.70
4	258	268	15–26	8 12	10.41	0.95	0.77	0.12	0.17	1.18	1.06	0 65	0 55	3 55	3 13	0 89	0.66	6.03	4.45	19.30	16.96
	±40	±44		±1.04	±0 54	±0 17	±0 10	±0.04	±0.04	±0 52	±0.30	±0 17	±0.09	±0.34	±0.39	±0.23	±0.04	±2.01	±1.84	±1.03	±1.15

* Mean per cent. The measure of variability is indicated by the standard deviation from the mean.

TABLE II

Effect of Parathyroid Extract upon Serum Calcium, Serum Inorganic Phosphorus, and Distribution, Retention, and Excretion of Radiophosphorus after Thyroparathyroidectomy

The experimental and the control animals are designated by the letters E. and C., respectively. One pair was used in each experiment.

Weight of animal		Dose of extract	Serum calcium		Serum inorganic phosphorus		Per cent of injected radiophosphorus per gm. tissue								Per cent of injected radiophosphorus recovered					
							Liver		Muscle		Femurs		Kidneys		Gastro-intestinal tract		Feces and contents of gastro-intestinal tract		Urine	
E.	C.	U S P	E	C.	E.	C.	E.	C.	E.	C.	E.	C.	E.	C.	E.	C.	E.	C.	E.	C.
gm.	gm.	units	mg. per cent	mg. per cent	mg. per cent	mg. per cent														
180	190	500	14 5	10.6	18 0	10 3	0.70	0 73	0 14	0.16	1 77	2.00	0.52	0 62	2 43	4.10	1.02	3.30	34.1	16.0
163	161	250	16 6	11.2	13.7	8 8	0.73	0 73	0 16	0.24	2 30	2.93	0 60	0 62	2.95	4 18	3 80	3.79	27.1	18.8
164	162	125	13.1	10.6	9.2	12.5	0.91	0 79	0.17	0.22	2 42	2.86	0.50	0 62	3.51	4.46	2.10	2.80	34.2	17.6
238	245	125	15 1	11.4	11.4	9.3	0.60	0 64	0.11	0 15	1 73	2 19	0.47	0 53	3.39	4 49	4 10	4 00	34 3	19 6
183	179	50	12.2	10.7	10.1	9 0	0.51	0.61	0.17	0 17	3 11	3.66	0.52	0.55	3 72	4.06	2.64	3.20	24 6	13.5
170	170	20	10 1	10.5	11 5	11 5	0.57	0.70	0 18	0 17	3 02	3.11	0.53	0 54	4.59	4.67	3.41	3.90	21 6	15 6

phate. 18 hours later these animals were decapitated. The activity values of their urine only are shown in Table IV.

Series V—The animals used in these experiments received the stock diet *ad libitum* before the experiment, but during the experimental period they had access to water only. Rat A-1 (Table V) was injected with 250 units of parathyroid extract 2 hours after bilateral nephrectomy. 20 minutes after the injection of parathyroid extract, Rat A-1 and its nephrectomized and normal controls (Rats B-1 and C-1) were each injected

TABLE III

Effect of Parathyroid Extract upon Serum Calcium, Serum Inorganic Phosphorus, and upon Retention and Excretion of Radiophosphorus in Fed and Fasted Thyroparathyroidectomized Rats

The experimental and the control animals are designated by the letters E and Tpc., respectively. 10 U. S P units were administered in each case

Group No	No of pairs	Weight of animal		Serum calcium		Serum inorganic phosphorus		Per cent* of radiophosphorus recovered			
								Femurs, per gm		Urine	
		E	Tpc.	E	Tpc	E.	Tpc	E	Tpc	E	Tpc
		gm	*gm*	*mg per cent*	*mg per cent*	*mg per cent*	*mg per cent*				
1†	5	170	169	8 6	8 6	12 7	12 7	3 05	3 59	14 5	8 3
		±20	±22	±0 6	±1 0	±3 7	±2 8	±0 35	±0 38	±4 9	±3 4
2‡	4	182	178	7 0	6 1	13 4	14 6	3 28	3 43	14 9	5 1
		±56	±52	±0 3	±0 5	±0 8	±1 7	±0 83	±0 76	±5 5	±1 3

* Mean per cent The measure of variability is indicated by the standard deviation from the mean

† Food intake unrestricted before and during experimental period

‡ Food intake restricted to 10 gm daily for the 3 days just before the experiment, and during the period fasted

with a tracer dose of phosphate. 18 hours later Rat A-1 received a second injection of 250 units of parathyroid extract. Rats A-2 and A-3 (Table V) received four injections of parathyroid extract. The first dose of 20 units was administered 2 hours after they were subjected to bilateral nephrectomy. 20 minutes after the injection of the parathyroid extract these animals and their respective controls were each injected with labeled phosphate. The other three doses of parathyroid extract were administered to Rats A-2 and A-3 at 2, 4, and 12 hours after the injection of the tracer dose of phosphate. 24 hours after the administration of the labeled phosphate all of the animals were sacrificed. The activity values of the tissues and the two normal urines are shown in Table V.

TABLE IV

Effect of Parathyroid Extract* upon Distribution, Retention, and Excretion of Radiophosphorus after Thyroparathyroidectomy

All values are expressed as per cent of the administered radiophosphorus recovered (for blood, liver, muscle, femurs, per gm.). E. represents the experimental animal, Tpc. the thyroparathyroidectomized control.

No. of pairs	Weight of animal E.	Weight of animal Tpc.	Time after injection of P³³	Blood E.	Blood Tpc.	Liver E.	Liver Tpc.	Muscle E.	Muscle Tpc	Femurs E.	Femurs Tpc	Urine E.	Urine Tpc.
	gm	gm	hrs.										
1	158	158	1	0.35	0.38	0.95	1 59	0 16	0.13	3.20	3 59	4 64	0.96
1	157	153	1			1.84	1 86	0 17	0 16	3 09	3.84	2 29	1 32
1	167	165	1			1 87	1 61	0 22	0 18	3 82	3.86	2.27	2.90
1	166	169	1	0 38	0 41	1.74	1 55	0 14	0 14	3.23	3 76	15 17	3 08
(1)	158	162	1	0 31	0 43	2.01	1.90	0.16	0 20	2.61	2 57	12.76	0.60
1	162	169	3	0 29	0 31	1.43	1.71	0.12	0 13	3 70	4.42	12.53	2 02
1	180	189	3			1.67	1 93	0 19	0.14	2.58	2.93	3.92	0.48
1	180	202	5			0.73	1 39	0.13	0.14	2.94	3.39	15.15	5.05
(1)	174†		5			1.57		0.12		3.48		17.05	
1	192	189	5	0.20	0 29	1 18	1.35	0.11	0 14	3 16	3.76	10.62	2 65
1	154	154	18			0 60	0.71	0 09	0 09	2.28	2 13	12 45	3.90
1	173	182	18			0 86	0.91	0.13	0.16	2 86	3 93	15.50	2.89
9‡	177§	173§	18									9.12§	7.90§
	±15	±14										±2 70	±1.50

* The experimental animal of each pair of rats, except those of the two in parentheses, received 5 U S P XII units of parathyroid extract. Each of the experimental animals of these two pairs received 2 5 units.

† Control found dead in cage.

‡ The nine pairs of normal animals received the same dietary treatment as the other animals shown in this table.

§ Mean values and standard deviation from the mean.

TABLE V

Distribution, Retention, and Excretion of Radiophosphorus after Bilateral Nephrectomy and Administration of Parathyroid Extract

All values are expressed as per cent of administered radiophosphorus recovered (blood, liver, muscle, femurs, per gm.). A represents the experimental animal, B the nephrectomized control, and C the normal control.

Rat No.	Weight	Parathyroid extract injected	Blood	Liver	Muscle	Femurs	Stomach and small intestine	Large intestine	Feces and contents of intestines	Urine
	gm	U.S.P units								
A-1	95	2 × 250	0 25	1 89	0 30	9.31	3.31	0.51	1.09	
B-1	97		0 20	1 96	0.26	9.00	4 27	0 43	1.52	
C-1	100		0.17	1.29	0.32	6.65	2.26	0.35	3.09	25.3
A-2	86	4 × 20	0 25	1 34	0 43	7.00	4.95	0 75	3.43	
B-2	90		0.24	1.45	0 38	7.97	4.57	0.56	2.07	
C-2	93		0 22	1.29	0.25	5.29	2 43	0.35	3.91	19.8
A-3	74	4 × 20	0.27	2.51	0.49	8.03	4.03	0.80	2.03	
B-3	74		0.26	2.46	0.51	9.48	5.70	0.69	2.53	

Results

The results of the first experiments (Table I) show that durirg the 24 hours immediately following thyroparathyroidectomy the serum calcium of the rats dropped below 8 mg. per cent, thus indicating parathyroid insufficiency. On the other hand, the related, or simultaneous, change in the urinary excretion of phosphorus was equally pronounced. During the 18 hour experimental period of the 1st day following the operation, the experimental animals excreted only about 4.9 per cent of the administered radiophosphorus in the urine and 2.6 per cent in the feces, while their controls excreted by these routes 17.9 and 3.6 per cent, respectively. The excess radiophosphorus retained by the experimental animals did not accumulate in any one site, since the activity measurements show that the average specific content of P^{32} in the femurs, liver, muscle, and kidneys of the experimental animals was in each instance about 30 per cent higher than the average specific content of P^{32} in the corresponding tissues of the controls. Furthermore, calculations based on the data in Table I, and Donaldson's (10) data on the weights of the parts and organs of the rat, indicate that the amounts of the radiophosphorus taken up by the various tissues of the animals operated on depended only on their relative mass and phosphorus content.

Under the conditions of these experiments, a readjustment of the several intrinsic and extrinsic factors affecting phosphorus excretion appears to have occurred without the supervention of manifest tetany. Rats which were injected with a tracer dose of phosphate 1 to 25 days after thyroparathyroidectomy were able to excrete much larger amounts of radiophosphorus by both the urinary and the fecal route than animals similarly tested during the first 24 hour postoperative period. 7 to 26 days after thyroparathyroidectomy, the kidneys, the femurs, and particularly the muscle showed a decreasing specific content of P^{32}. During this period the specific content of P^{32} in the muscle dropped about 30 per cent below the control values. In the femurs and kidneys, however, although the specific content of P^{32} dropped sharply, it did not drop below the control values. On the other hand, the retention of radiophosphorus in the liver decreased very slowly. Our data reveal that 15 to 26 days after thyroparathyroidectomy the specific content of P^{32} in the livers of the experimental animals was still about 20 per cent higher than that of the controls. From these observations it appears that a slower uptake, or a lower retention, of the administered radiophosphorus in the muscle of the experimental animals, than that in their controls, must have accounted for the greater urinary and fecal excretion of radiophosphorus shown by the former 15 to 26 days after the operation.

During the second 24 hours after thyroparathyroidectomy, when the kidneys still showed a decreased ability to excrete radiophosphorus, the urinary excretion of radiophosphorus was greatly increased by the injection of parathyroid extract. Thyroparathyroidectomized rats (Table II), which received from 20 to 500 units of parathyroid extract 1 hour before the injection of labeled phosphate, excreted by the urinary route amounts of the radiophosphorus which were approximately 44 to 113 per cent larger than the amounts excreted by their normal controls. In the animals in which 125 to 500 units of parathyroid extract gave rise to hypercalcemia, there was usually a lower specific content of P^{32} in the various tissues than occurred in the corresponding tissues of the controls. Single doses of 20 or 50 units of parathyroid extract were less decisive in their effect on the specific content of P^{32} in the tissues, but the larger dose appears to have caused a lower initial uptake of radiophosphorus by the femurs, or to have increased the movement of radiophosphorus therefrom. Of the various observed effects of parathyroid extract upon the calcium and phosphorus metabolism of these animals, the elevation in the serum calcium, the reduced specific content of P^{32} in the femurs, and the accelerated urinary excretion of radiophosphorus were usually the most prominent.

A comparison of the effects of the action of 10 units of parathyroid extract on fed and fasted thyroparathyroidectomized rats (Groups 1 and 2, Table III) is of particular interest. In Group 1, the serum calcium level of both the experimental animals and their controls was 8.6 mg. per cent at the end of the 18 hour experimental period, whereas in Group 2 the average serum calcium of the experimental animals was 7.0 mg. per cent and that of their controls 6.1 mg. per cent. The animals of Group 1 showed no obvious tetany at the end of the experimental period, but all of the animals of Group 2 exhibited slight, moderate, or severe tetany. The serum inorganic phosphorus of the experimental animals of neither Group 1 or 2 was appreciably different from that of their respective controls. On the other hand, the effect of parathyroid extract on the urinary excretion of radiophosphorus was quite definite. The experimental animals of Group 1 excreted approximately 14.5 per cent of the administered radiophosphorus and their controls excreted about 8.3 per cent. In the case of the animals of Group 2, the experimental animals excreted 14.9 per cent of the administered radiophosphorus, while their controls excreted only 5.1 per cent.

The easily demonstrable increase in the urinary excretion of radiophosphorus following the injection of 10 units of parathyroid extract indicated the feasibility of testing the action of smaller doses. Also, the foregoing experimental results indicated the desirability of establishing as closely as possible the interval of time during which a relatively small amount

of parathyroid extract influences the urinary excretion of injected radio-phosphorus. Accordingly, the following experiments were carried out with these considerations in mind.

It will be observed that in the case of the thyroparathyroidectomized rats (Table IV) which received 5 units of parathyroid extract the urinary excretion of radiophosphorus after 1 hour was in one instance approximately as high as that observed after 18 hours. Four experimental animals, that were sacrificed after 1 hour, had excreted amounts of the radiophos-phorus representing 2.27 to 15.20 per cent of the tracer dose. In the same period their controls excreted 0.96 to 3.08 per cent of the tracer dose. After 18 hours, two experimental animals had excreted 12.45 and 15.50 per cent, respectively, of the administered radiophosphorus as compared with excretion values of 3.90 and 2.89 per cent by their respective controls. 3 to 5 hours after the injection of the labeled phosphate, three of the four experimental animals had excreted amounts of the injected radiophos-phorus comparable to the amounts excreted by the experimental animals that were sacrificed after 18 hours. The extraordinary sensitivity of these thyroparathyroidectomized rats to parathyroid extract is clearly evident when their excretion values of radiophosphorus are compared with those of normal animals which received 5 units of parathyroid extract. The average amount of the administered radiophosphorus excreted in the urine of nine normal rats (Table IV) during the 18 hour experimental period was 9.12 per cent as compared with 7.90 per cent for the controls.

Indicative of the small amount of parathyroid extract actually required to accelerate the urinary excretion of administered radiophosphorus by the thyroparathyroidectomized rats were the results obtained after the injection of 2.5 units of parathyroid extract. It will be observed (Table IV) that one of the two experimental animals had excreted 12.76 per cent of the tracer dose of phosphate within 1 hour, and that the other excreted 17.05 per cent of the tracer dose within 5 hours.

The increased urinary excretion of radiophosphorus by the thyro-parathyroidectomized animals, following the injection of 2.5 or 5 units of parathyroid extract, does not appear to have affected the uptake or the retention of radiophosphorus in the muscle (Table IV). On the other hand, the slightly lower specific content of P^{32} in most of the blood and bone samples of the experimental animals than that seen in their controls may be attributed to the increased urinary excretion of radiophosphorus, which appears to have proceeded simultaneously with the absorption and distribution of the radiophosphorus. It seems apparent that parathyroid extract retarded the accumulation of the administered radiophosphorus in the femurs to a large extent, if not entirely, by a direct action on the kidneys, whereby the urinary excretion of radiophosphorus was accelerated.

This opinion is strengthened by some observations that were made on nephrectomized animals (Table V) that received injections of parathyroid extract and a tracer dose of phosphorus. The results of these experiments show that in the absence of the kidneys large doses of parathyroid extract had no effect upon the uptake, retention, or fecal excretion of radiophosphorus.

DISCUSSION

It is generally accepted that the fall in serum calcium, the immediate decrease in the urinary excretion of phosphate, and the retention of phosphate in the blood following thyroparathyroidectomy are due to the loss of parathyroid function, and not to the removal of the thyroid. Consequently, in the interpretation of our data we have considered these acute effects of thyroparathyroidectomy as indicative of parathyroid insufficiency alone.

Heretofore, there has been a paucity of information concerning the sites of phosphate retention in the body of the parathyroidectomized animal, as well as some question whether the increased retention of phosphate, frequently manifested by a high serum inorganic phosphorus level, is the cause or the effect of the decreased excretion of phosphate. It has been shown in our experiments that immediately following the loss of parathyroid function in fed rats (Table I) the urinary excretion of administered radiophosphorus was sharply decreased, and that the amount of the radiophosphorus retained in the various tissues apparently depended upon their relative mass and phosphorus content. Also, it was observed in the rat in the absence of the kidneys (Table V), but with the parathyroids presumably unimpaired, that the amounts of radiophosphorus found in the various tissues after the injection of a tracer dose of phosphate presented a similar pattern to that observed in rats with intact kidneys, but with no parathyroids. From these observations, it appears that the increased retention of radiophosphorus in the various tissues of the thyroparathyroidectomized rats was caused by an increased uptake of the radiophosphorus, due to a decreased ability of the kidney to excrete the labeled phosphate at the time it was being absorbed and taken up by the tissues. That the failure of parathyroid function, so far as phosphorus metabolism is concerned, first occurs in the kidneys is further indicated by the increase in the urinary excretion of radiophosphorus and the normal retention pattern of the unexcreted portion in the tissues when sufficient amounts of parathyroid extract were administered to thyroparathyroidectomized rats (Table II).

The remarkable effectiveness of small doses of parathyroid extract in accelerating the urinary excretion of radiophosphorus in the fed or the

fasting thyroparathyroidectomized rat indicates an increased sensitivity of the kidney to parathyroid hormone. In the experiments in which 5 units, or less, of parathyroid extract were used, the injections of the extract and the tracer dose of phosphate were so spaced as to assure the probable appearance of a small amount of parathyroid hormone in the blood at the time the absorption of the labeled phosphate began. From the results of these experiments (Table IV), it appears likely that the larger part of the radiophosphorus found in the urine was excreted during the short interval in which the labeled phosphate was being absorbed. The lower specific content of P^{32} in most of the livers and femurs of the hormone-treated rats than that seen in their controls strongly suggests that the uptake of radiophosphorus was reduced by the concurrent increase in the urinary excretion of radiophosphorus. That parathyroid extract increases the urinary excretion of phosphate by causing a diminished reabsorption of phosphate by the renal tubules has been affirmed by Harrison and Harrison (11), and denied by Fay, Behrmann, and Buck (12).

The reason for the marked sensitivity of the kidney of the thyroparathyroidectomized rat to parathyroid extract is not entirely clear. Conceivably, a slower turnover of phosphorus in the tissues, caused by the ether anesthesia, may have facilitated the action of parathyroid extract in promoting the urinary excretion of the injected radiophosphorus. In any case, the practical significance of an increased sensitivity of the kidney to parathyroid extract is obvious. It is believed that the data presented in this connection provide the basis for the development of a method of detecting extremely small amounts of parathyroid hormone.

Shelling and Asher (13) have presented evidence that the loss of the regulatory action of parathyroid hormone on the urinary excretion of phosphorus may be partly offset by a diet containing about 0.5 gm. per cent of phosphorus and a larger amount of calcium, whereby the excretion of phosphorus is shifted from the urine to the feces. Their best results were obtained with a diet low in phosphorus and relatively high in calcium, in a ratio of about 0.25:1. The diet used in our experiments contained about 1.54 gm. per cent of calcium and 0.98 gm. per cent of phosphorus. Even with this high phosphorus diet, we observed in rats (Table I), 2 to 5 days after thyroparathyroidectomy, a marked tendency of injected radiophosphorus to shift from the urinary to the fecal route of excretion. Further indication that the excess calcium of the ingested diet was a factor in promoting the excretion of phosphorus by way of the feces is seen again in the excretion values of radiophosphorus shown by rats (Table I) 15 to 26 days after thyroparathyroidectomy. It will be observed that the amount of radiophosphorus found in the feces of these animals was about 35 per cent larger than the amount found in the feces of their normal controls.

Although the excess calcium of the ingested diet appears to have facilitated the fecal excretion of radiophosphorus by the thyroparathyroidectomized animals, this alone does not account for the increased excretion of radiophosphorus.

A compensatory change in the kidney and a slower turnover of phosphorus in the tissues, arising from the absence of the thyroid or the parathyroids, may also have favored the excretion of injected radiophosphorus. The increased facility with which injected radiophosphorus was excreted by rats 2 to 5 days after thyroparathyroidectomy would appear to be due to a compensatory change in the kidney, and not to a slower turnover of phosphorus in the tissues. However, the data suggest that within a few days after thyroparathyroidectomy a slower turnover of phosphorus in the muscle may have caused a decreased uptake of the injected radiophosphorus, and hence favored a greater excretion of radiophosphorus. It will be observed that 7 to 12 days after thyroparathyroidectomy the muscle tissue of the rats showed a lower specific content of P^{32} than that of the controls. Furthermore, a decreased retention of radiophosphorus in the muscle tissue is also seen in the animals which were tested 15 to 26 days after the operation. At that time these animals also showed a higher urinary and fecal excretion of radiophosphorus than their controls, despite the fact that their tissues, with the exception of muscle, showed about the same, or a higher, specific content of P^{32} than the corresponding tissues of the controls. These observations suggest that the lower retention of radiophosphorus in the muscle tissue was due to a decreased uptake of radiophosphorus, which may have occurred as the result of the failure of parathyroid function in the muscle. This opinion is strengthened by the evidence presented by Brown and Imrie (14) and Imrie and Jenkinson (15) that the parathyroid glands are concerned with the metabolism of creatine phosphate. These authors observed that the creatine phosphate in the muscle of thyroparathyroidectomized cats, in which the symptoms of tetany were well marked, tends to be lower than in normal cats, and that following stimulation of the muscle the rate of resynthesis of creatine phosphate was very much slower than that observed in normal cats. Furthermore, they observed that the creatine phosphate and the orthophosphates in the muscle of thyroparathyroidectomized cats treated with parathyroid extract resemble the normal.

SUMMARY

The distribution, retention, and excretion of administered radiophosphorus (P^{32}) has been determined both in untreated thyroparathyroidectomized rats and untreated bilaterally nephrectomized rats, and in rats similarly operated on after the injection of parathyroid extract.

1. During the first 24 hours following thyroparathyroidectomy, the urinary and fecal excretion of injected radiophosphorus decreased sharply. The amounts of the administered radiophosphorus retained in the various tissues appeared to depend only upon their relative mass and phosphorus content.

2. Within 48 hours after thyroparathyroidectomy, the urinary and fecal excretion of administered radiophosphorus increased markedly, and 15 to 26 days after the operation exceeded the control value. As the excretion of radiophosphorus increased, its retention in the tissues decreased, but only in the case of muscle did the retention value of radiophosphorus drop below the control value.

3. A reversal of the marked retention of injected radiophosphorus in the tissues during the first 24 hours after thyroparathyroidectomy and an increased urinary excretion of the administered radiophosphorus occurred when doses of 20 to 500 units of parathyroid extract were injected.

4. The prompt action of 5 units of parathyroid extract in promoting the urinary excretion of administered radiophosphorus in the thyroparathyroidectomized rat is interpreted as evidence of a direct action of parathyroid hormone upon the kidney.

5. After bilateral nephrectomy, the administration of parathyroid extract had no effect upon the distribution, retention, or excretion of radiophosphorus.

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THE RÔLE OF AMINO ACIDS IN THE RENAL TUBULAR SECRETION OF AMMONIA*

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During the course of 24 hours the normal individual excretes the equivalent of 300 to 500 ml. of 0.1 N acid in combination with ammonia. This quantity, however, represents only a fraction of the renal capacity to excrete ammonia, for the diabetic in severe acidosis may excrete more than 10 times this amount. The origin and mode of excretion of urinary ammonia have been disputed for a number of years. In 1921 Nash and Benedict (1) first showed conclusively that it is formed in the kidney from some precursor in the blood. In 1940 Walker (2) demonstrated directly that the cells of the lower two-thirds of the distal convoluted tubules of the amphibian kidney synthesize and actively secrete ammonia into the tubular urine. It is probable that the distal segments of the mammalian tubule function in a similar manner. Most investigators have considered urea to be the plasma precursor of urinary ammonia (3, 4); others have implicated amino acids (5) or the amide nitrogen of plasma proteins (6). In general these views have been based more on speculation than on conclusive evidence. Recently, however, Van Slyke and his associates (7), studying the renal extraction ratios of the several nitrogenous constituents of the plasma, have clearly demonstrated that in the acidotic dog some 60 per cent of urinary ammonia is derived from the amide nitrogen of plasma glutamine, and the remaining 40 per cent from plasma amino nitrogen. Urea does not contribute to urinary ammonia (8, 9). The synthesis of ammonia by the cells of the renal tubules from both glutamine and amino acid signifies that these precursors must undergo deamidation and deamination within those cells. A glutaminase, present in kidney tissue (10), catalyzes the degradation of glutamine to glutamic acid and ammonia. At least three amino acid oxidases, likewise present in kidney tissue, catalyze the deamination of certain amino acids to their corresponding keto acids and ammonia. These are glycine oxidase (11), *d*-amino acid oxidase (12), and *l*-amino acid oxidase (12, 13). Presumably one concern of these enzymes in-the living animal is the synthesis and secretion of ammonia by the kidney. This presumption would be considerably strengthened if it were demonstrated that the excre-

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tion of ammonia by the intact animal presented with an excess of glutamine or amino acid could be correlated with the activity *in vitro* of these enzymes on their various substrates. And indeed Van Slyke and his associates have demonstrated that the intravenous administration of glutamine to the acidotic dog markedly enhances the rate of ammonia excretion (7). Bliss has observed, in the case of amino acids, that the administration of alanine and leucine produce a similar effect (14). Glutamine, leucine, and alanine yield ammonia when exposed *in vitro* to renal glutaminase and amino acid oxidases respectively. In the present study a representative number of amino acids were administered to the acidotic dog by intravenous infusion. The rate of ammonia excretion was measured at a series of comparable plasma concentrations of amino acid. It was found that the capacity of these amino acids to alter ammonia excretion *in vivo* is well correlated with their susceptibility to oxidative deamination *in vitro* in the presence of renal deaminative enzyme systems. The evidence upon which this statement is based is presented below.

<div align="center">EXPERIMENTAL</div>

Experiments have been performed on two female mongrel dogs, trained to lie quietly while loosely restrained on a comfortable animal table. Urine collections were made with an indwelling catheter. The collection periods were 10 minutes long, and when the urine flow was less than 10 ml. per minute the bladder was washed with distilled water and the washings added to the original urine. Analyses were performed on arterial plasma, a blood sample being drawn from the femoral artery at the mid-point of each collection period. A portion of blood was stored on ice in a 2 ml. Van Slyke stop-cock pipette for subsequent pH measurement; a second portion was centrifuged in a completely filled and tightly stoppered ampul for the determination of plasma carbon dioxide; and a third portion was centrifuged, without special precautions, for other analytical procedures described below. Constant plasma levels of creatinine and increasing plasma levels of amino nitrogen were obtained by infusions administered intravenously at a rate of 10 ml. per minute. During each experiment plasma amino nitrogen was increased stepwise from normal levels of 3.4 to 4.5 mg. per cent to levels of 20 to 25 mg. per cent by infusions of increasing amino acid concentration.[1] Each infusion was allowed to run for 20 minutes before the experimental periods were begun to allow the plasma concentration of amino acid to attain approximate equilibrium. During the 2 days prior to each experiment 500 to 600 ml. of 3 per cent ammonium chloride were given by stomach tube to produce acidosis. 1 hour before the start of each experi-

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[1] Plasma amino nitrogen levels much above 25 mg. per cent tend to cause vomiting and depression of renal function, as evidenced in a decreased glomerular filtration rate and renal blood flow.

ment, water was given by stomach tube in amounts of 50 ml. per kilo of body weight to insure adequate hydration.

Chemical Methods

Amino nitrogen determinations were made on picric acid filtrates of plasma and urine after removal of urea with urease by the ninhydrin gasometric method of Van Slyke, Dillon, MacFadyen, and Hamilton (15), as modified by Hamilton (16). Creatinine was determined on iron filtrates of plasma (17) and diluted urines by the method of Folin and Wu (18). All colorimetric analyses were made with an Evelyn photoelectric colorimeter. The carbon dioxide content of plasma was determined manometrically by the method of Van Slyke and Neill (19), and the pH of whole blood was measured with a condenser type of glass electrode without exposure to air. The latter measurements were made at room temperature and corrected to $37°$ ((20) p. 166). Ammonia in urine was determined by aeration into boric acid and titration with 0.01 N H_2SO_4. Urinary titratable acid was measured by titrating urine samples electrometrically to the observed pH of the blood, with the glass electrode as the measuring instrument.

Determination of Renal Function

The creatinine clearance was used as a measure of the rate of filtration of plasma through the glomeruli. Since it seemed desirable to determine completely the reabsorptive capacity of the kidney tubules for the various amino acids as well as the ability of these same amino acids to effect ammonia elimination, the quantity of amino nitrogen filtered, excreted, and reabsorbed was measured during each experiment. The quantity of amino nitrogen filtered through the glomeruli was calculated as the product of the glomerular filtration rate in ml. per minute and the plasma amino nitrogen concentration in mg. per ml. The quantity of amino nitrogen excreted was calculated as the product of urine flow in ml. per minute and the urine amino nitrogen concentration in mg. per ml. The difference between the quantity filtered and that excreted is the quantity reabsorbed by the tubules.

The choice of amino acids used in this study was determined by availability, solubility, and lack of toxicity. It would have been preferable to use only the natural isomers, but resolved forms of all amino acids were not available in the quantities required by the experiments.

Results

Each of the following amino acids[2] was studied in duplicate experiments performed on the same two dogs: (1) *monoaminomonocarboxylic* acids,

glycine, *dl*-alanine, *l*(−)-leucine; (2) *diaminomonocarboxylic* acids, *l*(+)-arginine hydrochloride and *l*(+)-lysine hydrochloride; (3) *monoaminodicarboxylic* acids, *l*(+)-glutamic acid and *dl*-aspartic acid; (4) a hydrolysate of

TABLE I

Effect of Amino Acid Administration on Renal Tubular Functions of Ammonia Secretion and Amino Nitrogen Reabsorption

Total time	Arterial plasma				Glomerular filtration rate	Urine		Rate of excretion		Amino nitrogen		
	pH	BHCO₃	Amino N	Creatinine		Flow	pH	Ammonia	Titratable acid	Filtered	Excreted	Reabsorbed

Experiment 1   Dog 11, *dl*-alanine

min	*7 31*	*mm per l*	*mg per cent*	*mg per cent*	*ml per min*	*ml per min*		*m eq per min*	*m eq per min*	*mg per min*	*mg per min*	*mg per min.*
105–115	7 31	13 6	4 24	31 3	53 6	6 6	5 18	0 073	0 059	2 27	0 06	2 21
115–125			4 22	29 6	56 2	5 7	4 94	0 076	0 072	2 37	0 05	2 32
140–150	7 31	13 7	8 16	30 0	62 9	5 6	5 13	0 137	0 070	5 14	1 09	4 05
150–160			8 50	27 5	68 4	5 7	5 10	0 141	0 071	5 82	1 35	4 47
175–185			13 5	27 6	73 7	6 5	5 18	0 165	0 071	9 95	3 46	5 49
185–195			14 9	28 3	72 7	6 9	5 27	0 170	0 071	10 8	4 18	6 66
210–220	7 31	14 7	19 9	28 1	77 4	8 2	5 48	0 179	0 066	15 4	7 03	8 37
220–230			20 0	28 3	82 1	9 2	5 51	0 182	0 069	16 4	8 00	8 42

Experiment 2   Dog 11, *l*(+)-glutamic acid

165–175	7 30	12 4	4 62	29 4	62 1	7 0	4 78	0 081	0 080	2 87	0 08	2 79
175–185			4 57	29 4	61 2	6 9	4 76	0 086	0 088	2 80	0 07	2 73
200–210	7 30	12 1	6 77	28 3	59 0	6 9	4 84	0 088	0 098	4 00	0 80	3 20
210–220			7 63	28 3	66 1	5 3	4 88	0 088	0 096	5 04	1 21	3 83
235–245			11 8	27 4	68 6	6 0	5 14	0 085	0 088	8 10	3 61	4 49
245–255			13 2	27 8	72 5	7 0	5 22	0 089	0 089	9 57	5 01	4 56
275–285	7 27	13 4	19 7	28 3	67 6	7 9	5 36	0 079	0 092	13 3	8 53	4 79
285–295			20 6	28 4	66 2	7 6	5 37	0 075	0 092	13 6	9 23	4 40

casein with added tryptophane. The data obtained in two representative experiments on Dog 11 are presented in Table I. In Experiment 1, following two initial control periods, *dl*-alanine was administered at such a rate as to increase the plasma amino nitrogen concentration from its control value of 4.2 to 20.0 mg. per cent. The degree of acidosis in this animal was moderately severe, as is indicated by plasma pH values of 7.31 and bicar-

bonate contents of 13 to 15 mM per liter. As a consequence of this acidosis the urine was highly acid and ammonia was excreted in the control periods at the elevated rate of 0.073 to 0.076 milliequivalent per minute. The administration of alanine caused an immediate and sharp rise in the rate of ammonia excretion. Doubling the plasma amino nitrogen concentration approximately doubled the rate of ammonia elimination. Further increase in plasma amino acid concentration, although increasing ammonia production, did so to a progressively diminishing degree.

Experiment 2 was performed in a fashion identical with that of Experiment 1 with the exception that $l(+)$-glutamic acid was infused in place of dl-alanine. In order to avoid rapid changes in acid-base balance the glutamic acid in the infusions was half neutralized.[3] It is apparent that the degree of acidosis, the control rates of ammonia excretion, control levels of plasma amino nitrogen, and the range of increase in plasma amino nitrogen concentration were nearly the same in the two experiments. Yet it is equally apparent that the kidney did not utilize $l(+)$-glutamic acid for the purpose of increasing ammonia excretion. In fact the rate of ammonia excretion remained constant, within the range of accuracy of the experimental procedures, throughout the experiment. The data of these two experiments assume added significance when viewed in the light of the observation of Blanchard et al. (13) that $l(+)$-glutamic acid is not oxidatively deaminized by l-amino acid oxidase, whereas dl-alanine is so affected by both d and l forms of that enzyme present in kidney tissue (12, 13).

The data obtained in sixteen experiments similar to those presented in Table I are summarized in Figs. 1 to 6. In the upper portion of each figure the rate of excretion of ammonia over and above that of the two control periods is plotted against the plasma concentration of amino nitrogen. In the lower portion the rate of reabsorption of amino nitrogen over and above that of the two control periods is similarly plotted against the plasma concentration of amino nitrogen. Thus, in each pair of graphs values for the control periods were arbitrarily set at zero. This appeared to be justifiable because of the close agreement between the control observations of the several experiments [4]

[3] Preliminary experiments indicated that half neutralization of glutamic and aspartic acids produced minimal disturbances of the acid-base balance, i e neither expansion nor depletion of the alkali reserve, changes that would have seriously disturbed the experiments

[4] The extent of the acidosis in the several experiments was relatively constant; the bicarbonate content of the plasma was maintained within a range of 11 to 14 mM per liter The control values for plasma amino nitrogen were similarly constant within limits of 3 5 and 4 6 mg per cent Amino acid excretion was negligible in control periods of all experiments, and the rate of ammonia excretion varied between 0 060 and 0 090 milliequivalents per minute.

In Figs. 1 and 2 are grouped the data obtained in experiments in which monoaminomonocarboxylic acids were administered. Arbitrarily included in this group are the data obtained on administration of casein hydrolysate. It is evident from the upper graphs of Figs. 1 and 2 that glycine, *dl*-alanine, $l(-)$-leucine, and casein hydrolysate all significantly increase the rate of ammonia excretion in the acidotic dog, glycine and alanine being somewhat
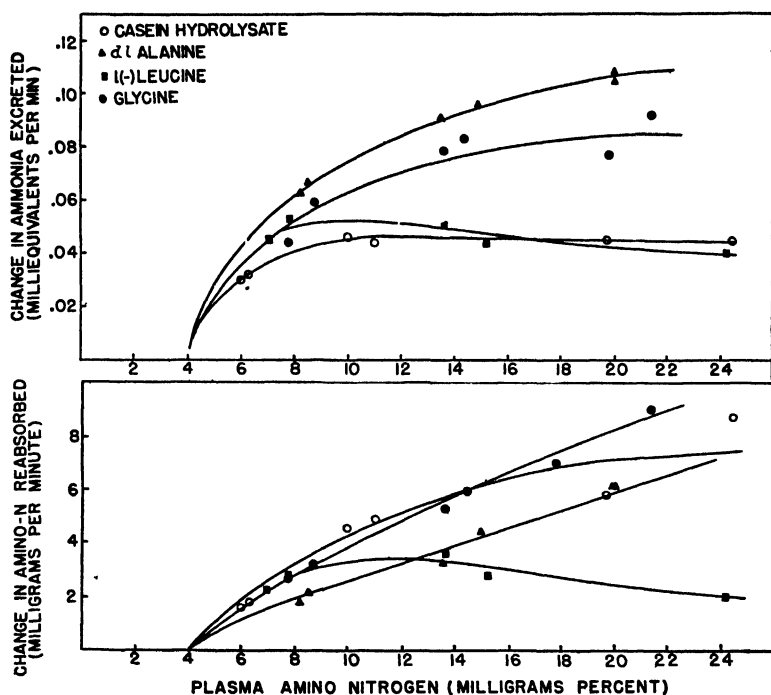
FIG. 1. Monoaminomonocarboxylic acids. Rate of tubular secretion of ammonia and tubular reabsorption of amino nitrogen as functions of plasma amino nitrogen concentration   Dog 11

more effective at all plasma concentrations. The most rapid increase in ammonia excretion is effected by moderate elevations in plasma concentration, *i.e.* to 8 or 10 mg. per cent of amino nitrogen. Further increases in the plasma concentration of glycine and alanine are accompanied by further increases in ammonia excretion, but the rate steadily declines and a plateau is reached at plasma concentrations of 20 to 25 mg. per cent. Leucine and casein hydrolysate tend to produce their maximum stimulating effects on ammonia excretion at somewhat lower plasma concentrations. Each of these amino acids is subject to oxidative deamination *in vitro* in the presence
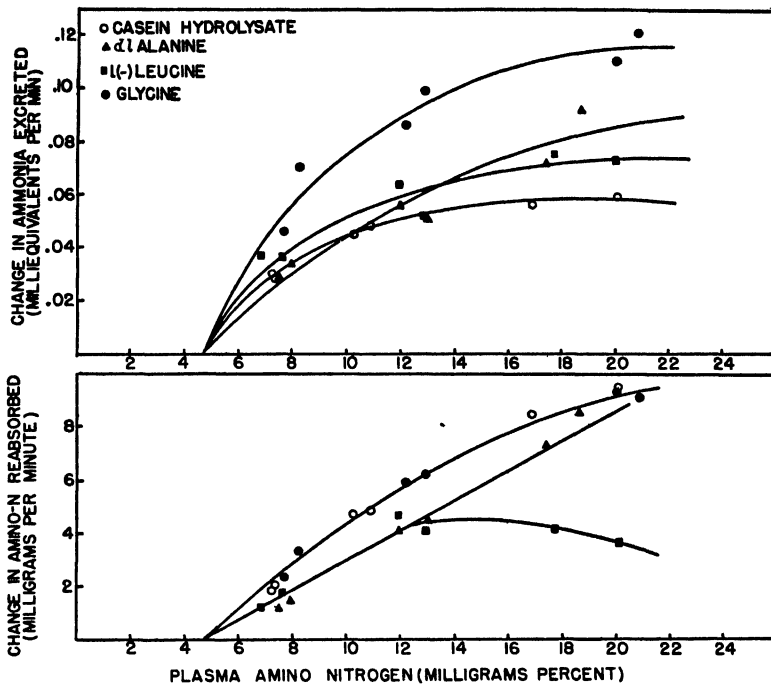
Fig 2 Monoaminomonocarboxylic acids. Rate of tubular secretion of ammonia and tubular reabsorption of amino nitrogen as functions of plasma amino nitrogen concentration. Dog 6.
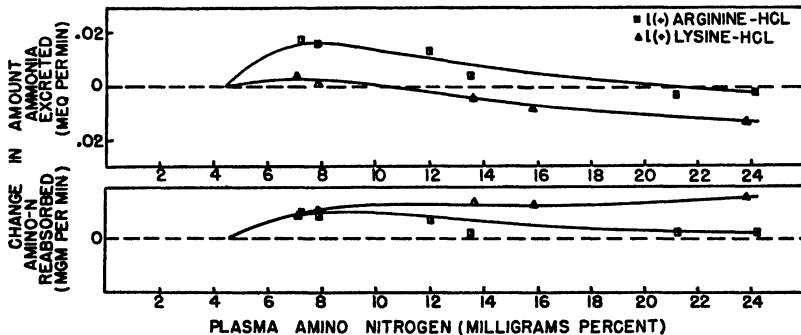
Fig 3 Diaminomonocarboxylic acids Rate of tubular secretion of ammonia and tubular reabsorption of amino nitrogen as functions of plasma amino nitrogen concentration Dog 11.

of renal deamirases: glycine by glycine oxidase (11), $l(-)$-leucine by l-amino acid oxidase (13), and dl-alanine and certain components of casein hydrolysate by both d- and l-amino acid oxidase (12, 13).

In the upper graphs of Figs. 3 and 4 are grouped the data obtained in experiments in which the diaminomonocarboxylic acids, $l(+)$-arginine and $l(+)$-lysine, were infused. The change in ammonia excretion following the
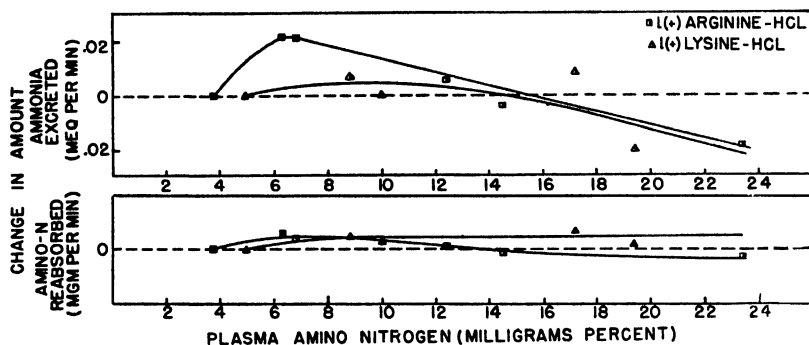
FIG 4 Diaminomonocarboxylic acids Rate of tubular secretion of ammonia and tubular reabsorption of amino nitrogen as functions of plasma amino nitrogen concentration Dog 6
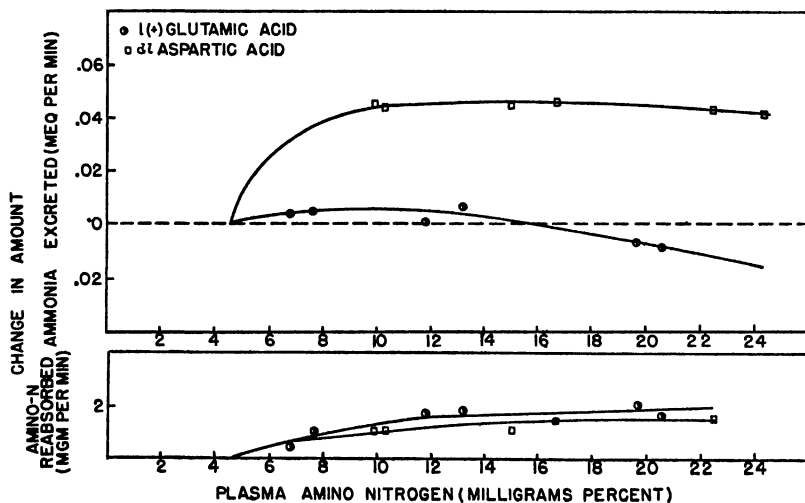
FIG 5 Monoaminodicarboxylic acids. Rate of tubular secretion of ammonia and tubular reabsorption of amino nitrogen as functions of plasma amino nitrogen concentration. Dog 11

administration of these amino acids is negligible. The small increment in ammonia excretion early in both of the arginine experiments is of doubtful significance. No change which was out of the range of experimental error of the procedure occurred in the lysine experiments. These results agree

well with the demonstration by Blanchard *et al.* (13) that the *l* forms of the dibasic amino acids do not undergo oxidative deamination under the influence of *l*-amino acid oxidase.

In the upper graphs of Figs. 5 and 6 are grouped the data obtained in experiments in which the monoaminodicarboxylic acids, *l*(+)-glutamic acid and *dl*-aspartic acid, were infused. No significant change occurred in ammonia excretion in either glutamic acid experiment. However, the changes in the aspartic acid experiments are significant and comparable in magnitude to the increases effected with leucine and casein hydrolysate. *l*-Amino
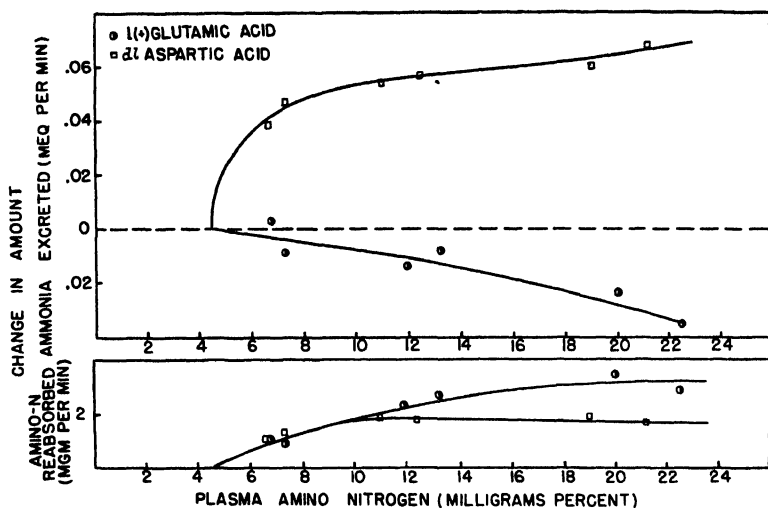
FIG 6 Monoaminodicarboxylic acids   Rate of tubular secretion of ammonia and tubular reabsorption of amino nitrogen as functions of plasma amino nitrogen concentration   Dog 6

acid oxidase does not affect either the *d* or *l* forms of the dicarboxylic acids *in vitro* (13). This agrees with the fact that *l*(+)-glutamic acid caused no increase in ammonia excretion. On the other hand *d*-amino acid oxidase does effect the oxidative deamination of *d*-aspartic acid *in vitro* (12); therefore the increased rate of ammonia excretion in the aspartic acid experiments probably resulted from the oxidative deamination of the *d* component of the mixture by the *d*-amino acid oxidase of the kidney. If this is so, it is then evident that the correlation between the activity *in vitro* of the renal enzymes in deamination of amino acids and the stimulation *in vivo* of ammonia synthesis and secretion by those same amino acids is a perfect one for the limited series of amino acids studied.

DISCUSSION

There is evident in the data presented above a correlation between the activity of the renal enzymes, glycine oxidase, *d*-amino acid oxidase, and *l*-amino acid oxidase, acting *in vitro* on their respective substrates and the capacity of these same substrates to enhance ammonia elimination *in vivo* in the acidotic dog. This observation supports the general thesis that amino acid oxidases in the kidney of the intact animal are concerned, among other things, with the synthesis and secretion of ammonia and hence play a rôle in the renal regulation of acid-base balance.

There has further appeared in these experiments some degree of correlation between the capacity of the kidney to utilize an amino acid for the synthesis of urinary ammonia and the capacity of the renal tubules to reabsorb that same amino acid.[5] This correlation is evident on comparing Experiments 1 and 2 of Table I. Thus at plasma concentrations of 20 mg. per cent some 8 mg. of amino nitrogen were reabsorbed per minute in the alanine experiment, in which the rate of ammonia elimination was more than doubled. On the other hand, only 4 mg. of amino nitrogen were reabsorbed per minute in the glutamic acid experiment, in which the rate of ammonia elimination remained nearly constant. This correlation is also apparent in the graphs of Figs. 1 to 6. Thus it is evident that glycine and alanine, which considerably augment ammonia elimination, are reabsorbed by the renal tubules to a relatively greater degree. Leucine, somewhat less active in stimulating ammonia secretion, is reabsorbed to a lesser degree. Lysine, arginine, and glutamic acid do not increase ammonia production and are poorly reabsorbed by the renal tubules. Two obvious exceptions are evident. Casein hydrolysate, which is reabsorbed to an extent comparable with glycine and alanine, is less effective than either in augmenting ammonia secretion; *dl*-aspartic acid, although poorly reabsorbed, approximately doubles the rate of ammonia elimination. The reasons for these exceptions are not immediately obvious. However, the basis for the correlation, in so far as one exists, might be found in one of two possible explanations. First, different physical properties of the various amino acids may determine the different rates of penetration of those acids into the cells of the renal tubules, both from the tubular urine and the peritubular blood. On the other hand, some common chain of intracellular reactions might participate in both the processes of tubular reabsorption of amino acid nitrogen and tubular synthesis of ammonia. Since the evidence has implicated

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[5] This correlation by no means implies that the amino acid which is reabsorbed is that which is deaminized to form urinary ammonia. The quantity of amino nitrogen reabsorbed always greatly exceeds that which is deaminized in ammonia synthesis.

renal amino acid oxidases in the tubular synthesis and secretion of ammonia, it is tempting to assume that these same enzymes are likewise involved in the tubular reabsorption of amino acid nitrogen. It has been shown that amino acids undergo chemical reactions of a first order type during their tubular reabsorption (21). The exact nature of these reactions is obscure. However, it would appear logical to assume that amino acid oxidases are involved. This assumption is consistent with the fact that deamination is a necessary preliminary to most reactions in which amino acids are involved (22, 8, 23).

In vitro, the rate of deamination of amino acids by a limited quantity of amino acid oxidase is a curvilinear function of the concentration of the substrate. At high concentrations a limiting rate of deamination is attained which is characteristic for each amino acid. Furthermore, rate of deamination is directly proportional to enzyme concentration (13). One may note in Figs. 1, 2, 5, and 6 that, *in vivo*, the rate of secretion of ammonia is similarly a curvilinear function of plasma amino nitrogen concentration, and that at high plasma concentrations a limiting rate of excretion is attained which is characteristic for each amino acid. It might be assumed that the rate of ammonia elimination at high plasma levels of amino acid is limited by the rate of deamination of substrate by a fixed, limited quantity of enzyme. Within the limitations of these data this assumption is consistent with the observed facts.

It has been repeatedly observed that, if a given quantity of acid is administered to an animal each day over a period of several days, the quantity of ammonia eliminated in the urine progressively increases with time until the ammonia output becomes equivalent to the acid intake (24). This may represent a purely functional response of the kidney to progressive depletion of the alkali reserve of the body. It may, however, represent a type of biochemical compensation expressed as an increase in the intracellular concentration of amino acid oxidase and glutaminase. Such an increase in enzyme concentration might enable the kidney to increase its ammonia production at normal plasma concentrations of glutamine and amino acid. That the plasma concentrations of these two substrates are unchanged in acidosis is evident from the work of Van Slyke *et al.* (7) on glutamine, and the present study on amino acids.

SUMMARY

A representative number of amino acids have been administered to acidotic dogs by intravenous infusion. The rate of tubular secretion of ammonia and the rate of tubular reabsorption of amino nitrogen have been studied at a series of comparable plasma concentrations of each amino acid. It has been demonstrated that glycine, *dl*-alanine, *l*(−)-leucine, *dl*-aspartic acid,

and a hydrolysate of casein produce an increase in the rate of ammonia secretion in the acidotic dog. $l(+)$-Arginine, $l(+)$-lysine, and $l(+)$-glutamic acid are without such an effect. It has been shown that the capacity of these amino acids to alter ammonia secretion *in vivo* is correlated well with their susceptibility to oxidative deamination *in vitro* by renal amino acid oxidases. This has lead to the conclusion that renal amino acid oxidases are concerned in the living animal with the synthesis of ammonia by the kidney and hence play an important rôle in the renal regulation of acid-base balance It has been further noted that there exists a correlation between the extent to which an amino acid can increase ammonia secretion and the extent to which that same amino acid is reabsorbed by the renal tubules. The basis for this correlation is not definitely known, but it has been proposed that either the physical properties of the several amino acids or a common chain of intracellular reactions might similarly limit both tubular processes

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PREPARATION OF DIPHOSPHOPYRIDINE NUCLEOTIDE*

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A recent publication by Sumner *et al.* (1) discusses the need for a simplified method of producing diphosphopyridine nucleotide (DPN), a compound needed in studies of many enzyme systems. These authors call attention to the complexity of available methods and present a new procedure, which though somewhat simplified, uses expensive materials and gives very low yields. The potency of their preparations is difficult to estimate, since they base it upon an enzymatic assay of the DPN content of the yeast used as source material, without reference to any standard. Data presented for nitrogen, carbon, hydrogen, and phosphorus are not those of pure DPN and are acknowledged to be a poor criterion of purity. While they present spectrophotometric data for wave-lengths of 210 to 300 $m\mu$ with a peak at 260 $m\mu$ these are characteristic of other adenine nucleotides generally found as impurities in DPN preparations. No data are given for the absorption of the reduced form at 340 $m\mu$

It is the purpose of this paper to present a method which has been in use in our laboratories for over a year, during which time it has served to supply needs for some 25 gm. of DPN preparations at low cost. Pressed bakers' yeast appears to be the most generally available and cheapest source material[1] A method described by Williamson and Green (2) provides a simple means of obtaining a DPN concentrate in good yield. Williamson and Green report obtaining, by this method, material assaying 65 per cent and possessing no phosphorus-containing impurities. Using their procedure either exactly as described or as slightly modified in our final method, we consistently obtain preparations assaying 27 to 30 per cent and containing considerably more phosphorus and pentose than can be ascribed to the DPN content.

Procedure for Crude Preparation

A vessel containing four liters of distilled water is heated, with mechanical stirring, in such manner that heat is supplied rapidly. When the tempera-

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[1] It is important that this be fresh, since yeast obtained when 2 days old gave approximately twice the yield given by that which had been stored 1 week in the refrigerator

ture has reached 92°, addition of the crumbled yeast is begun. It is added as rapidly as possible with the temperature maintained at 90–92°. When 10 pounds of yeast have been added, the heat is withdrawn and the vessel is immersed in cold water and rapidly cooled to room temperature. The yeast suspension is filtered on Büchner funnels with suction, with a filter aid such as Hyflo Super-Cel. A pressure filtering device is used for this operation when available.[2] The filtrate, 4000 to 4300 ml., is treated with 10 per cent of its volume of 25 per cent basic lead acetate solution, $Pb(OAc)_2 \cdot Pb(OH)_2$, and the resulting precipitate filtered out and discarded. The filtrate from the lead treatment is adjusted to pH 6.5 with acetic acid, chilled, and 50 ml. of 25 per cent silver nitrate solution stirred in. The resulting precipitate is permitted to settle in the refrigerator and the bulk of the liquid decanted off. The slurry is centrifuged in 250 ml. bottles and the supernatant discarded. The silver precipitate is washed three times with successive portions of distilled water, each approximately 3 times the volume of the packed precipitate. Washing is carried out in the centrifuge bottles. The precipitate is stirred up in 50 to 70 ml. of water and decomposed with hydrogen sulfide. The silver sulfide is centrifuged, washed once with a small volume of water, and discarded. The supernatant and wash are filtered and aerated free of hydrogen sulfide. 5 volumes of cold acetone are added. The resulting precipitate, which contains the DPN, is centrifuged in the cold, the supernatant discarded, and the precipitate dried *in vacuo* over sulfuric acid. The yield at this point is 1200 to 1500 mg. of material assaying 27 to 30 per cent DPN.

While this amount of material can be carried through the rest of the procedure with essentially the same recoveries, it is almost as readily carried out with 5 or 10 times as much material. Consequently this crude preparation is allowed to accumulate before further purification is begun.

Procedure for Second Stage of Purification

The crude cozymase (10 gm.) is dissolved in 775 ml. of 0.1 M acetic acid and to it are added 25 ml. of 25 per cent lead acetate, $Pb(OAc)_2$, and 2 liters of 95 per cent alcohol. The solution is chilled to 0°. The resulting precipitate is centrifuged and discarded. The supernatant is treated with 25 per cent silver nitrate in excess (approximately 50 ml.) and the silver salt of DPN centrifuged down in the cold. After the precipitate is washed once in the centrifuge bottle with approximately twice its volume of water, it is suspended in 100 to 150 ml. of water. Hydrogen sulfide is passed in to decompose the silver salt. This decomposition takes only a few minutes if one stoppers the bottle and shakes it once or twice during the gassing. The silver sulfide is centrifuged, washed once, and discarded.

[2] We have been able to facilitate greatly this operation with a Sparkler horizontal pressure filter.

Excess hydrogen sulfide is aerated off[3] and the solution is neutralized to pH 7.0 with sodium hydroxide, care being taken to avoid localized areas of high pH. The volume is made up to 400 ml. with distilled water and 72 gm. of charcoal[4] added. The suspension is shaken or stirred vigorously for 20 minutes, then filtered on a fritted glass filter with suction. Experiments with various ratios of charcoal and DPN demonstrated that under these circumstances the DPN is all adsorbed on the charcoal. The charcoal is washed, on the filter, with 300 ml. of 2 per cent trichloroacetic acid (to remove sodium ions) and with 100 ml. of water. These washes do not remove any DPN as shown by complete absence of pentose in the filtrates. The washed charcoal is suspended in 360 ml. of a 20 per cent pyridine[5]-80 per cent water mixture (volume) and stirred vigorously for 20 minutes. Then the charcoal is filtered off on the fritted glass funnel and the elution repeated. The two eluates are combined and concentrated under reduced pressure at 30–35° with a water aspirator. The pyridine keeps the pH within safe limits. When the volume has been reduced to 40 to 50 ml. (pH now 4.0 to 4.5), the solution is filtered to remove the last traces of charcoal and treated with 6 to 7 volumes of cold acetone. The precipitation is best carried out in small, successive portions in a 50 ml. centrifuge tube. This minimizes losses due to material adhering to the sides of the tube. When it has all been centrifuged down,[6] the precipitated DPN is dried *in vacuo* over sulfuric acid.

The dry material can be scraped from the tube and ground in a mortar. Since the material is hygroscopic, it is advisable to dry the powder again. The yield is now 3400 to 3800 mg. of white material assaying 63 per cent DPN, with consistent 80 per cent recoveries of the DPN originally present in the crude material.

Assay

Our preparations are first tested in a biological system requiring DPN, the malic dehydrogenase system (3). If amounts of the preparations corresponding to 50 to 200 γ of DPN are added per Warburg flask, an almost

[3] If at this point the DPN is precipitated with acetone and dried, the resulting preparation is 54 to 57 per cent DPN and the recovery of the DPN present in the starting material is approximately 90 per cent. These preparations, and the starting material, both exhibit, in biological systems, effects attributable to traces of heavy metals.

[4] Norit A decolorizing charcoal proved most suitable. This tends to have alkaline salts present in it as received, and it is best to wash it on a Büchner funnel with water and air-dry it before use

[5] Even the reagent grade pyridine that we obtain contains small amounts of colored material, which all appears in the final DPN preparation unless eliminated. Distillation from glass served to remove these undesirable impurities from the pyridine.

[6] More centrifuging is required when the DPN reaches higher potency than when it is in a very crude state as it was earlier in the procedure.

linear response is obtained. Larger quantities can be added to obtain a plateau on the oxidation rate. The latter gives an indication as to whether a preparation contains traces of heavy metals, since the accumulated effect of these is to lower oxidative rates.

Measurement of the absorption of the reduced form of DPN at 340 mμ as first used by Warburg and Christian (4) appears to be the most precise method of assay.[7] This was accomplished in our laboratory by use of a Beckman spectrophotometer. The details of this assay were worked out by Dr. Van R. Potter, with whose permission they are included in this manuscript.

The assay procedure is as follows: The DPN sample is made up as a 1 per cent solution. To a 16 × 150 mm. tube is added 0.20 ml. of water + 0.50 ml. of fresh 1 per cent sodium bicarbonate, followed by 0.10 ml. of the 1 per cent DPN preparation (1000 γ). 2 ml. of a freshly prepared 3 per cent solution of sodium hydrosulfite in 1 per cent sodium bicarbonate are prepared in a small tube with cautious stirring to avoid aeration. A 0.20 ml. aliquot of the sodium hydrosulfite solution is added to the tube containing the DPN sample, mixed in gently, and allowed to stand 20 minutes at room temperature without further agitation. Now to the sample tube are added 9.0 ml. of a 1 per cent sodium bicarbonate-1 per cent sodium carbonate solution. The mixture is aerated for 5 minutes to remove excess hydrosulfite. Aeration is by means of compressed air passed through a water wash. The sample is used to obtain absorption at 340 mμ in the Beckman spectrophotometer.

A reading can be taken with a second aliquot not receiving the reduction treatment. This provides a correction for absorption of the oxidized form or any impurities. Our final preparations have a negligible absorption at 340 mμ before reduction. Some of the cruder preparations show approximately one-tenth as high an absorption before as after reduction.

For calculation of the DPN concentration, the constant given in War-

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[7] The original procedure was not proposed as an assay but has been frequently used for assay purposes without its being stated whether the reduction was carried out in Warburg flasks with 95 per cent N$_2$-5 per cent CO$_2$ in the gas phase. In the present procedure the ratio of Na$_2$S$_2$O$_4$ to DPN has been increased, the use of the gas mixture has been eliminated, and the reaction is carried out at room temperature. The concentration of Na$_2$S$_2$O$_4$ is such that exclusion of oxygen is unnecessary. If *fresh* bicarbonate is used, the pH remains in the correct range of 7.4 to 7.9 during the reduction without having 5 per cent CO$_2$ in the gas phase. On standing, bicarbonate solutions lose CO$_2$ and the pH rises to above 8.0. In this pH range, the reduction product absorbs at 340 mμ but its maximum is at 360 mμ and it can be observed visually as a yellow color, it is believed to be the free radical or half reduced compound, and unlike the fully reduced compound it is reoxidized by air in the presence of sulfite, so that the E_{340} decreases on standing (5) (V. R. P.)

burg's review as the average of the best preparations was used (6). The value for E_{345} was given as 1.3×10^7 cm.^{2} per mole which converts to 8.5×10^3 cm.^{2} per gm. The E_{340} value is about 2 per cent higher than the E_{345} but the constant has been used as given. On this basis, the concentration in micrograms per ml. is given by multiplying the E_{340} (1 cm.) by 118, and since the samples are made up at a concentration of 100 γ per ml. this value is also the per cent purity of the sample.

The following variations in the assay method were found to have no effect on the result: (1) increased amounts of sodium hydrosulfite up to 12 mg.; (2) incubation of the sample, after reduction, for 15 to 90 minutes; (3) incubation at 38° instead of at room temperature (20–25°); (4) washing out the sodium bicarbonate solution with 95 per cent nitrogen-5 per cent carbon dioxide and bubbling this gas through the samples during incubation; (5) variation in the bicarbonate from 0.20 to 0.70 ml.; (6) variation in the DPN sample from 0.10 to 0.50 ml.; (7) aeration beyond 5 minutes.

DISCUSSION

The preparation of DPN described above requires relatively small quantities of solvents and depends chiefly upon cheap chemicals, such as lead salts and charcoal. The whole procedure can be accomplished within 2 to 3 days while other work is carried on. The main portion of the work, the accumulation of crude DPN by the slightly modified Williamson-Green method, can be carried out by undergraduate part time helpers or technical assistants with satisfactory results

The use of charcoal adsorption has been described for DPN purification by Jandorf (7), and now more recently by Sumner *et al.* However, Jandorf used it earlier in the purification procedure and accomplished elution by shaking with an amyl alcohol-water mixture for long periods. We were unable to obtain anything approaching complete elution by this means, as apparently was the case in the method of Sumner *et al.* Procedures involving fractionations with lead and alcohol have been used by Warburg and Christian (4) and by Schlenk (8) for DPN fractionations.

The criticism has been offered (1) that the spectrophotometric assay would be subject to error because of the possible presence of inactivated DPN, which would be measured but have no biological activity. The assay procedure described here is as applicable to our crude preparations as to our best purified material. This is indicated by the relationship between the results of spectrophotometric and biological methods. When potency according to the spectrophotometric procedure increases 2-fold or more, the activity in the biological system (malic dehydrogenase) increases proportionately

SUMMARY

A relatively simple procedure is described for obtaining diphosphopyridine nucleotide from bakers' yeast in yields of 515 to 715 mg. of preparation per 10 pounds of yeast. The preparations are white, amorphous powders showing no trace metal effects in biological systems and assaying 63 per cent DPN by the measurement of reduced form at 340 mμ. A description of the assay procedure is included.

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A STUDY OF THE PURIFICATION AND PROPERTIES
OF RICIN

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Ricin, the highly toxic, hemagglutinating protein of the castor bean, has always been of great interest to the chemist and the immunologist (1–4), partly because it may readily be obtained in considerable quantities. Earlier methods of preparation were carried out before the development of modern physicochemical and immunochemical methods for characterizing proteins and data are therefore lacking on the purity and homogeneity of the products obtained.

In the present report a summary is given of a portion of a study carried out during 1943–45 in consultation and collaboration with other laboratories engaged in parallel investigations which, it is hoped, will soon be published. Since results and materials were freely exchanged, some of the data included here were obtained on material from other laboratories, as indicated in the text.

EXPERIMENTAL

Purification of Ricin—A partially purified sample of ricin (Lot A) was supplied by Dr. O. H. Alderks of The Procter and Gamble Company. It had been prepared according to Dr. A. H. Corwin of Johns Hopkins University by extraction of pressed castor beans with water at pH 3.8, precipitation of the toxin by saturation with sodium chloride, solution of the precipitate in water, and reprecipitation at pH 8 by saturation with sodium sulfate. Most of this material was obtained from castor beans without selection as to color. Other samples which had been prepared from white, gray, reddish gray, or black beans were identical in all properties examined.

7.5 gm. of Lot A were dissolved in water and the insoluble matter was centrifuged off (71 mg. of N). The solution and washings (650 mg. of N), at a volume of 300 ml., were treated with Na_2SO_4 solution saturated at 37°. Precipitation was interrupted after 175 ml. had been added. The precipitate (Fraction B1) was centrifuged off and the supernatant treated with an additional 95 ml. of warm saturated Na_2SO_4 solution. The precipitate (Fraction B2) was centrifuged off, leaving in the supernatant Fraction B3 which was precipitated by saturation with Na_2SO_4. Fractions B1

and B2 were each dissolved in water and freed from insoluble material. Fraction B1 was reprecipitated twice from a volume of 150 ml. by addition of 87.5 ml. of warm saturated Na_2SO_4 solution. The material in the supernatants from these reprecipitations was designated Fraction B1'. Fraction B2 was not further purified. Fraction B3 was taken up in a small volume of water and freed of a small amount of toxin (Fraction B3a) by addition of 45 per cent by volume of saturated Na_2SO_4 solution, leaving the supernatant,

TABLE I

Fractionation of Lot A by Sodium Sulfate

	Fraction B1	Fraction B1'	Fraction B2	Fraction B3a	Fraction B3b
Yield, *mg. N*	303	27	35	8	202
$[\alpha]_D$,* *degrees*	−28	−32	−32	−39	−59
Minimum hemagglutinating dose, γN	0 3	0 3	0 3	0 3	1000

Intraperitoneal toxicity for mice					
N injected					
γ					
0.01	0/3†	0/3		0/3	
0 1	3/3 <96 hrs ‡	3/3 <96 hrs	3/3 5 days	1/3 <95 hrs	
1	3/3 <24 "	3/3 <24 "	3/3 <70 hrs	3/3 <45 "	0/3
10	3/3 <24 "	3/3 <24 "	3/3 <20 "	3/3 <20 "	0/3
100					0/3
1000					0/3

* Optical rotation calculated for protein = $N \times 6.25$
† The number of deaths within 10 days to the number of mice injected
‡ Longest life period of any mouse which succumbed

Fraction B3b. Yields, optical rotation, hemagglutinating power, and toxicity for mice are given in Table I. It is evident that most of the toxin was concentrated in Fraction B1, which showed the lowest specific rotation. Fraction B3b, which contained about 30 per cent of the original soluble N, had less than 0.0001 of the toxicity of Fraction B1 and was readily dialyzable through cellophane membranes.

The toxicity of the fractions was estimated by intraperitoneal injection of 0.5 ml. of 10-fold serial dilutions of solutions standardized on the basis of nitrogen content. Female mice weighing 20 ± 3 gm. were used, three being injected with each dilution. Death or survival for 10 days was used as the end-point.

The hemagglutinating potency was measured as the minimum quantity of nitrogen giving definite agglutination $(+)$ of 0.2 ml. of a 4 per cent suspension of washed human group O erythrocytes. All tests were carried out in a volume of 1 ml. and were read after incubation at 37° for 1 hour.

Crystalline Ricin—Ricin was obtained in crystalline form by Kunitz[1] and also by Cannan.[2] Samples of three or four times crystallized material were made available through the courtesy of Dr. R. K. Cannan, Dr. M. Levy, and Dr. A. E. Benaglia of New York University.

Characterization of Crude and Purified Ricin—A comparison of the properties of crude ricin (Lot A), of Fraction B1, and of crystalline ricin is given in Table II. Both Fractions B1 and crystalline ricin showed a lower optical rotation than did the crude ricin; they were both electrophoretically and ultracentrifugally homogeneous, while Lot A showed two components in the ultracentrifuge and three components by electrophoresis. From sedimentation and diffusion, Fraction B1 was found to have a molecular weight of roughly 85,000 for a partial specific volume of 0.75. The sedimentation constant of crystalline ricin was slightly lower, leading to a molecular weight of 77,000, a value not considered significantly different from that of Fraction B1, since precise temperature control was not possible during the measurements of sedimentation. From these data a frictional ratio, $f f_0$, of 1.2 was found for Fraction B1 and the crystalline product, indicating a ratio of length to width of about 4 or 5 for a cylindrical or ellipsoidal shape and an unhydrated molecule. The mobilities of Fraction B1 and crystalline ricin were determined over a pH range of 4.0 to 8.5. The respective isoelectric points were calculated as 5.2 and 5.4 to 5.5, values that were practically the same.

A more precise comparison of the toxicity of these preparations was obtained by determining the minimum quantity of nitrogen (1 toxic unit) necessary to kill a 20 gm. mouse in 24 hours (modification of a method described by Dr. A. H. Corwin[3] of Johns Hopkins University). Ten mice were used for each sample to be assayed. Assays were carried out at New York University and at the University of Chicago through the courtesy of Dr. R. K. Cannan. Data in Table II indicate that samples of Lot A and Fraction B1 were only one-third and two-thirds as toxic as crystalline ricin.

The hemagglutinating potencies of Lot A and Fraction B1 were about the same within experimental error, 0.3 γ of N giving minimum agglutination of 0.2 ml. of a 4 per cent suspension of washed human erythrocytes when the tests were carried out in saline. The hemagglutinating power of

[1] Kunitz, M , personal communication.
[2] Cannan, R. K., personal communication.
[3] Corwin, A. H., personal communication

TABLE II

Properties of Ricin Preparations

	Lot A	Fraction B1	Crystalline ricin
$[\alpha]_D$, *degrees*	−41 5	−28 to −30	−26
Sedimentation constant, S, and relative concentrations of components	5 1 (67%) 0.8 (33%)	5.3* (100%)	4.8 (100%)
$D_{20} \times 10^7$ cm.^{2} per sec.		6.0†	
Mol. wt.‡		85,000	77,000§
f/f_0		1 2	1.2§
Approximate ratio of length to width‖		4–5	4–5

Electrophoretic mobility (u $\times 10^5$) and % components			
pH			
8 5	−1.0 (19%) −1.9 (77%) −4.1 (4%)	−2 2 (100%)	−2.2 (100%) −3.1 (Trace)
7.4	−1.3 (96%) −3.5 (4%)	−1 2 (100%)	−1.0 (100%)
6 05		−0 6 (100%)	−0 3 (100%)
5 0			+0.5 (100%)
4 9		+0 2 (100%)	
4 0			+2 1 (100%)
Isoelectric point, *pH*		5 2	5 4–5 5

Minimum hemagglutinating dose, $\gamma\,N$			
In saline	0.3	0 3	3–5
" 1:100 normal rabbit serum	0 3	0 3	0.5
Toxic unit per 20 gm. mouse, $\gamma\,N$¶	0 76	0 40	0.25
Crystalline ricin content from toxicity data, *i.e.* toxic N %	33	63–67	100**

Quantitative precipitin reaction with 1 0 ml. 1:20 pooled rabbit anti-Fraction B1 serum

N added	Extinction (Folin-Ciocalteau) of 1.0/2.5 ml. aliquot of washed ppt.		
γ			
0.5	0.067	0.103	0 09
1	0 117	0.175	0.175
2	0.206	0.36	0.335
3	0.276	0.51	0.47
5	0.43	0.70	0 68
7.5	0 593	0 84	0.87
10	0 71	0 97	0.93
15	0.79		

TABLE II—*Concluded*

N added	Extinction (Folin-Ciocalteau) of 1.0/2.5 ml aliquot of washed ppt.		
N to give extinction of 0.6, γ	7.6	4.0	4.0
Immunologically active ricin in preparation, %	53	100	100**
Detoxified N in preparation, %††	20	33–37	0**

* Average of 5.3, 5.4, 5.2, and 5.4 for several preparations at concentrations of 0.77, 0.53, 0.50, and 1.05 mg. of N per ml. At 2.0 mg. of N per ml. S was 5.0.

† Average of 5.98 and 6.08 for two preparations, corrected to two significant figures.

‡ For a partial specific volume of 0.75.

§ The diffusion constant is assumed to be the same as for Fraction B1.

‖ From f/f_0, for cylindrical or ellipsoidal shape.

¶ Determined at the University of Chicago.

** Assumed.

†† Immunologically active ricin minus crystalline ricin content from toxicity data.

crystalline ricin, however, was found to be only about one-tenth that of Fraction B1. Gilman and Phillips[4] observed that addition of normal serum increased the hemagglutinating potency of crystalline ricin. This was confirmed (Table II). By fractional precipitation with acetone it was found possible to obtain fractions with as little as one-fortieth of the hemagglutinating power of Fraction B1. Addition of serum or of 0.5 per cent of formalin restored hemagglutinating potency. These data suggest that hemagglutinating power, while a characteristic of ricin itself, is influenced by the presence of other substances.

Antisera to Fraction B1 and to crystalline ricin were prepared by immunization of rabbits with a formalinized toxoid prepared as follows: A solution containing 0.5 mg. of N of Fraction B1 per ml., buffered at pH 7.4 with 0.02 M phosphate and 0.15 M NaCl and containing 0.5 per cent formalin (1 part of 37 per cent formaldehyde solution to 200 parts of buffered ricin solution at pH 7.4), was kept at 37° for 5 days (in some instances 5 per cent formalin was used). This procedure resulted in about a 100- to a 1000-fold reduction in toxicity when 0.5 per cent formalin was used, and about a 1000-fold reduction in toxicity with 5 per cent formalin. Complete detoxication could not be obtained with formalin, as had previously been reported by Heymans (5). Marked loss of antigenicity occurred with formalin at pH 8.5 and above. Before use, the formalinized toxoid was precipitated by addition of enough 1 per cent alum to insure maximum flocculation. Because of the incomplete detoxication and the extreme susceptibility of the rabbit to ricin it was found necessary to

[4] Gilman, A., and Phillips, F. S., personal communication.

give each rabbit subcutaneous injections of 2.5, 5, and 5 γ of toxoid N
at 5 day intervals to induce some immunity before intravenous injections
were started. Each rabbit then received four intravenous injections
weekly for 4 weeks, as follows: two injections of 0.01 mg. of N, two of
0.03, four of 0.05, four of 0.15, and four of 0.50 mg. of toxoid N. Rabbits
were bled by cardiac puncture 5 days after the last injection. A later
course of intravenous injections could be given with 0 5 or 1 mg. of N
per injection. The animals became so resistant to the toxic effects of
ricin that immunization could be continued with equal doses of an alum
precipitate of undetoxified ricin.

Antiricin sera may be standardized by measuring their capacity to
neutralize the toxic effects of ricin on intraperitoneal injection into mice
or by assay of their inhibition of the hemagglutinating power of ricin.
Assays of the potency of a large number of rabbit antisera and of several
horse and goat antisera by both methods gave parallel results within
experimental error, providing strong support for the concept that hemag-
glutinating power and toxicity are properties of the same protein molecule.

A comparison of the immunochemical properties of Fraction B1 and
crystalline ricin was afforded by determination of their relative capacities
to precipitate with rabbit antiricin sera. These analyses were carried
out by addition of increasing amounts of a given preparation of ricin to a
measured volume of antiserum. After 1 hour at 37° and 1 week in the
ice box the precipitates were centrifuged off, washed twice in the cold
with saline, dissolved in water with the aid of 1 or 2 drops of 0.5 N NaOH,
and made up to 2.5 ml. Aliquot portions were analyzed by a modification
of the Folin-Ciocalteau tyrosine method as described by Heidelberger
and MacPherson (6) Extinction readings in the photoelectric colorimeter
plotted against the amounts of antigen nitrogen used gave a smooth curve
up to the point of complete precipitation of antibody. The curves obtained
with different preparations of ricin could be compared with respect to
their capacities to precipitate antiricin by determining the ratios of the
amounts of antigen required to give the same extinction. From Table II
it is evident that equal amounts of Fraction B1 or crystalline ricin nitrogen
were of equal potency, within experimental error, in precipitating antiricin
from an antiserum to Fraction B1. Similar results were obtained with
an antiserum to crystalline ricin. The extinction values obtained with
equal amounts of Lot A, however, were uniformly lower. Comparison of
the amounts of Lot A and of crystalline ricin or Fraction B1 nitrogen re-
quired to give an extinction reading of 0.6 indicates that 7 6 γ of N of Lot A
were required in contrast to 4.0 γ of N of fraction B1 or crystalline ricin.
With respect to its content of material immunochemically reactive as ricin,
Lot A would be only 4.0/7.6 or 53 per cent pure. Comparisons made at
any extinction reading in the antibody excess range may be used.

After removal of the specific precipitates, supernatants were divided in half and tested for the presence of antibody or antigen. With the antiserum to Fraction B1 as well as with antiserum to crystalline ricin, it was found that a given supernatant did not contain both antigen and antibody, even when crude products such as Lot A were used. Supernatants contained excess antigen or antibody, and in some instances neither antigen nor antibody could be detected. As demonstrated by Kendall (7), this provides strong evidence that the antisera contained only antibody to a single antigen, in this instance antibody to crystalline ricin or its immunological equivalent.

Although crystalline ricin and Fraction B1 were immunochemically identical on a nitrogen basis, in addition to having the same electrophoretic and ultracentrifugal properties within experimental error, Fraction B1 was only about two-thirds as toxic as crystalline ricin. This was taken to indicate that a portion of the ricin in Fraction B1 was non-toxic. Lot A also contained more ricin (53 per cent) by immunochemical analysis than could be accounted for by toxicity assays. A number of other crude ricin-containing fractions were analyzed by both methods and, in each instance, it was found that about one-third to one-half of the immunochemically measured ricin was non-toxic.

Accordingly, it was considered of interest to try to establish whether this non-toxic form of ricin existed in the intact castor bean or whether it was a result of chemical treatment in the course of purification. A number of different extracts of castor beans were assayed for their content of material immunochemically reactive as ricin and by Dr. A. E. Benaglia for toxicity and heat-coagulable protein. The results are given in Table III. Samples 272 and O were obtained from intact castor beans. The hulls were removed and an aqueous extract was prepared. Samples 217, 218, and M were extracts of castor beans pressed to remove oil; Sample 217 had been defatted with carbon tetrachloride-petroleum ether. In all instances, even in the aqueous extracts of shelled castor beans, ratios of toxic N to immunologically reactive N ranged from 0.46 to 0.64, indicating the presence of considerable non-toxic ricin. In these extracts the immunologically active nitrogen accounted for most of the heat-coagulable protein.

Since crystallization effects a separation of the toxic and non-toxic forms of ricin, it could be anticipated that the mother liquors, after removal of as much crystalline ricin as possible, should contain a lower proportion of toxic N to immunologically reactive N than the crude products. Sample 216, obtained from Dr. R. K. Cannan, Dr. M. Levy, and Dr. A. E. Benaglia, was such a mother liquor. The data in Table III show that the ratio of toxic to immunologically active N in this product was only 0.32, as compared with values of 0.46 to 0.64 for the unfractionated samples.

Estimation of immunologically reactive N by the quantitative precipitin method has been useful in following enzymatic digestion of ricin by pepsin

TABLE III

Correlation of Immunologically Active and Toxic Nitrogen in Ricin-Containing Crude Fractions of Castor Beans

Sample No. . . .	272	O	217	218	M	216	Crystal-line ricin
Total N, *mg. per ml.*	3.38	1.14	4.46	2 74	5 38	12.35	
Coagulable N, *mg. per ml*	0 92		1.58	0 72	2 04	10 32	
	(27%)	(23%)	(34%)	(26%)	(38%)	(84%)	100%
T.u.,* γ N per 20 gm. mouse†	2 2	2 6	2.6	2 6	1 9	1.6	0.35
$100 \times \dfrac{t\ u.,\ crystalline}{t\ u\ ,\ sample}$ = % toxic N	15 5	13.4	13	13	18	21	100‡

Quantitative precipitin reaction with 1 0 ml. 1:20 pooled rabbit anti-Fraction B1 serum

N added	Extinction (Folin-Ciocalteau) of 1 0/2 5 ml. aliquot of solution of washed ppt.						
γ							
0.5							0 09
1							0.175
2							0.335
2.5	0.101	0.113	0.135	0.119	0 113	0 315	
3							0 47
5	0.226	0 255	0.243	0 233	0 228	0 538	0.68
7.5	0 328	0 350	0 368	0 294	0 373	0.705	0 87
10	0.429	0 48	0.47	0 42	0.475	0 81	0 93
15	0 581	0 62	0 63	0 545	0 575	0 97	
20	0 695	0 74	0 735	0 655	0 735	1 14	
30	0.84	0 89	0 895	0 805	0 89	1.30	
40	0 98	1 07	1 04	0.95	1.05	1 40	
N to give extinction of 0 6, γ	15.8	14 5	14	17 2	14.4	6.0	4.0
Immunologically active N, % of total N	25	28	28 5	23	28	66	100‡
Ratio, toxic N to immuno-logically active N	0 62	0 48	0.46	0 57	0 64	0.32	1.0

* Toxicity unit.
† Determined at New York University.
‡ Assumed.

and trypsin. Digestion of ricin by pepsin at pH 4 or by trypsin at pH 7.4 or 8.5 was found to take place slowly, 1 to 2 weeks being required for the appearance of 40 to 50 per cent of products of low molecular weight. The

data in Table IV show that the disappearance of substance immuno-
chemically reactive as ricin corresponded closely to the appearance of
material of low molecular weight and to loss of toxicity.

TABLE IV

Digestion of Crystalline Ricin by Pepsin and Trypsin

Total N precipitable by	Peptic digest	Control	Tryptic digest	Control
Trichloroacetic acid, %	47	100		
Half saturation with Na$_2$SO$_4$, %	47	100	56	98
Non-dialyzable N, %			58.5	100

Quantitative precipitin curves of digested and untreated materials with 1.0 ml.
1:20 dilution of pooled rabbit antiricin serum

N added	Extinction (Folin-Ciocalteau) of 1 0/2 5 ml. aliquot of solution of washed ppt.			
γ				
1	0.088	0.154	0.093	0.193
2	0.141	0.305	0.192	0.384
4	0.302	0.568	0.368	0.65
6	0 40	0.75	0 466	0.825
8	0.56	0 875	0 613	0.94
10	0.68	1.03	0.715	1.10
15	0.83	1.15	0.87	1.19
20	0.99	1.22		
N to give extinction of 0.6, γ	8.8	4.0	7.7	3.9
Immunologically active N, %	46	100	51	100
Toxicity for mice				
0.05 γ N injected			1/3 6 days	3/3 < 45–95 hrs.
0.10 " " "	2/3 < 70, 95 hrs.	3/3 < 45–70 hrs.		

After dialysis of the digested samples the non-dialyzable N showed 93 per cent
of the precipitating power of crystalline ricin with antiserum.

DISCUSSION

By fractional precipitation with sodium sulfate it is possible to obtain
a highly toxic protein (Fraction B1) which is electrophoretically, ultra-
centrifugally, and immunochemically homogeneous, and identical by
these criteria with samples of crystalline ricin.[1,2] However, Fraction B1 is
only two-thirds as toxic as the crystalline product. Assays were made of
a variety of crude extracts of castor beans and of partially purified products
for toxicity and for material immunochemically identical with ricin.
These showed that there was uniformly less toxic nitrogen present than

immunologically active ricin nitrogen. The amount of substance immunochemically identical with ricin accounted for most, if not all, of the heat-coagulable protein in these materials. It may, therefore, be inferred that the castor bean contains two proteins, one of which is toxic, the other non-toxic, but which are immunochemically, ultracentrifugally, and electrophoretically identical. The toxic ricin in the castor bean extracts and in purified products, including Fraction B1, accounts for about one-half to two-thirds of the total ricin. It is possible that further studies at various stages in the development and germination of the castor bean might throw light on the relationships and mode of formation of toxic and non-toxic ricin.

The quantitative precipitin method could not be used to standardize antiricin sera on the basis of their content of specifically precipitable nitrogen, since ricin was found to precipitate normal serum protein as well as other proteins. The washed specific precipitates from antiricin sera consisted of ricin, antiricin, and coprecipitated normal serum protein, but this did not interfere with the use of calibration curves of antiricin sera for the estimation of the amounts of total ricin in various preparations.

Alternative possibilities to the existence of toxic and non-toxic ricin in the non-crystalline preparations have been considered but were rejected as contrary to the evidence. For example, if it is assumed that, although crystalline ricin precipitates the same amount of total nitrogen from antisera as does the less pure Fraction B1, the precipitate consists of more antibody and less non-specific protein, as might be expected, the antibody would be exhausted by less crystalline ricin than by Fraction B1. Actually the equivalence zone is found at the same level of added antigen in both instances. This also disposes of the possibility that crystalline ricin precipitates less antibody and more non-specific protein.

These studies provide an additional instance of the value of quantitative immunochemical methods, especially when used in conjunction with other physicochemical and biological techniques, in the characterization and estimation of proteins (for other studies cf. (8–10)).

SUMMARY

1. Highly toxic, hemagglutinating preparations of ricin can be obtained by fractional precipitation with sodium sulfate. These products are electrophoretically, ultracentrifugally, and immunochemically homogeneous and identical in these respects with crystalline ricin, but are only two-thirds as toxic. The toxic and non-toxic forms can be separated by crystallization.

2. Immunochemical and toxicity assays of crude extracts of castor beans indicate that these contain both forms of ricin.

3. Ricin was found to have an isoelectric point of about 5.2 to 5.5 and a molecular weight by sedimentation and diffusion of about 77,000 to 85,000 for an assumed partial specific volume of 0.75.

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THE APPLICATION OF FLAME PHOTOMETRY TO SODIUM AND POTASSIUM DETERMINATIONS IN BIOLOGICAL FLUIDS*

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The need for a rapid, accurate method to determine quantitatively Na and K present together in biological fluids has been met by the procedures to be described. The necessity of separating these elements prior to their determination by the various chemical methods usually employed has led to procedures which are often prohibitively tedious and time-consuming. The recent development of flame photometry has now made possible physical methods of analysis in which chemical separation of Na and K is unnecessary (1, 2).

Although we are dealing with Na and K determinations only, the principle of flame photometry originally used by Lundegardh (3–5), and modified and called the air-acetylene flame method by Cholak and Hubbard (6), can theoretically be applied to the determination of all the alkaline metals. At present the available commercial instruments employ a relatively low temperature flame which activates the ions rather than the metals themselves; the sensitivity of the photocells is such that the lines of the metals themselves would not be of sufficient intensity to measure, and suitable optical filters for other metals are not readily available.

The present report concerns the use of the Perkin-Elmer flame photometer (model 18)[1] in sodium and potassium determinations on blood, plasma, red blood cells, and urine.

Materials and Methods

The Instrument—The model 18 Perkin-Elmer flame photometer[2] was developed primarily for use in industry when rapid and accurate Na and K determinations on inorganic solutions are desired. The instrument consists

essentially of (a) an aspirator and atomizer operated by compressed air, (b) a modified Meeker burner, (c) two photocells with filters, (d) an amplifier, and (e) a galvanometer. Solutions are aspirated and atomized at a steady rate, rise into the flame of the burner where the elements are heated to incandescence, and the amount of light emitted at particular wavelengths is measured by a null point galvanometer (1). The light to the Na photocell passes through an optical filter whose highest per cent transmission is 5844 A (5465 to 6500 A), while the K photocell is covered by a filter whose peak transmission is 7716 A (7000 to 9240+ A).[3]
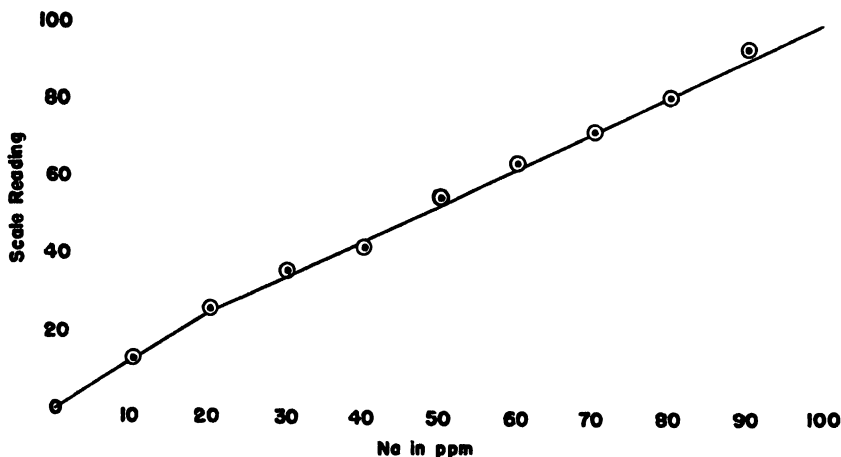
Fig 1 Calibration chart for Na (0 to 100 p p m) in the presence of 0 to 100 p p m of K

In practice the instrument is first calibrated with solutions of known ionic strength covering the range of concentrations expected in the unknown solutions Thus the zero point of the vernier controlling the null point galvanometer may be set with distilled water and the 100 per cent point with a solution containing 100 parts per million (0.01 mg. per cent) of Na by weight By making determinations on known solutions between these two points, calibration curves are constructed (Figs. 1 and 2) covering the range of concentrations expected in the unknown solutions to be tested

Although with suitable calibrations a concentration of Na as high as 10,000 p.p.m. (1 mg. per cent) can be determined, actual experience has revealed that the most satisfactory results are obtained on solutions containing less than 100 p.p.m. each of Na and K (1). Consequently, dilutions of blood, plasma, red cells, and urine are necessary before determinations are made

[3] Fraser, W. A., personal communication

Since the presence of a high concentration of one element may affect the readings made on another element, it is likewise important to use solutions for calibrating the instrument which contain approximately the same *relative* amounts of Na and K as are to be expected in the unknown.   In this regard, it has been shown (7) that a solution containing 1000 p.p.m. of Na will give a deflection amounting to 3 p.p.m. on the K photocell, while a solution containing 1000 p.p.m. of K will give a galvanometer deflection corresponding to 0.2 p.p.m. on the Na photocell.   At the concentrations of Na and K encountered in the procedures to be described, these errors are almost negligible; nevertheless it has seemed advisable to prepare the standards with this interference in mind.
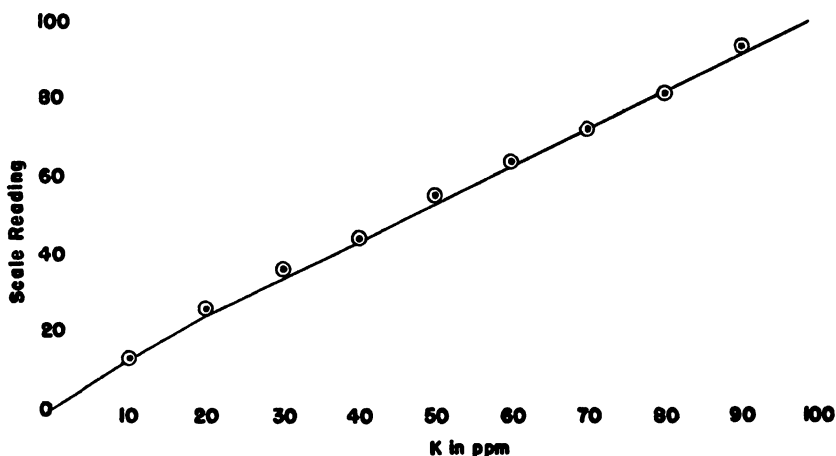
Fig 2   Calibration chart for K (0 to 100 p p m) in the presence of 0 to 100 p.p m of Na

Before each unknown solution is run through the instrument, the zero point and the 100 per cent point are checked with the standard solutions It has been found that running an "intermediate standard" (*i.e.*, some solution of known concentration between 0 and 100 per cent) is an additional check on the accuracy of the calibration.

The error encountered in flame photometric determinations on biological fluids is due to the fact that runs are made on diluted samples and consequently the small machine error (1 or 2 p.p.m) must be multiplied by a relatively large dilution factor (usually a factor of 5 or 6).   However, operation of the instrument in the manner described and making multiple (three or five) readings on each unknown will produce results which have an accuracy of ±4 per cent of the total amount of Na present and ±3 per cent of the total amount of K present (Table I).   Thus, the accuracy

of the flame photometric determinations of Na and K in blood, plasma, and urine approximates that of the microchemical methods (8). Owing to the introduction of the dilution factor in magnifying the machine error, it will be apparent that the recoveries of small amounts of added Na, for example, from relatively large amounts already present (Table I, first line), will result in sizable *recovery* errors. The solution of this problem may lie in "internal standard" flame photometry as described by Berry, Chappel, and Barnes (2).

TABLE I

Recovery of Na and K from Dog Plasma by Flame Photometry

The results, expressed in mg. per 100 ml., are the averages of fourteen determinations.

Present	Added	Total	Found	Average recovery	Per cent error
			Sodium		
367	8	375	375, 368, 368, 354, 361	365	−2 66
367	32	399	394, 396, 400, 425, 413	406	+1.75
367	98	465	451, 473, 465, 451, 413	457	−1.72
367	168	535	550, 538, 562, 535, 444	545	+1.87
367	221	588	600, 626, 575, 581, 585	593	+0.85
			Potassium		
7.7	24.3	32	31.3, 30.6, 30.6, 31.3	31.0	−3.13
17.0	81	98	91, 103, 101, 94	97	−1.02
17.0	148	165	163, 168, 172, 163	167	+1.21
17.0	205	222	231, 228, 231, 228	229	+3.15
17.0	253	270	281, 281, 281	281	+4.07
17.0	333	350	362, 362, 356	360	+2.86

The accuracy of reading the instrument, and consequently the accuracy of the determination, can be improved further by decreasing the ratio between the concentration and the scale (vernier) reading. Thus, if a solution containing 50 p.p.m. is used to set the zero point and one containing 80 p.p.m. is used to set the 100 per cent point (range setting), each of the 100 scale divisions will equal 0.3 p.p.m. instead of 1 p.p.m. as is the case when the zero is set with distilled water (0 p.p.m.) and a 100 p.p.m. solution is used to set the 100 per cent point. By thus reducing the concentration-scale division ratio, the accuracy can be increased to ±3 per cent of the amount of Na present and ±2 per cent of the amount of K present.

Preparation of Standard Solutions

The primary standards for flame photometric analysis are prepared by making separate solutions of sodium and potassium of 1000 p.p.m. concentration by weight. These solutions are diluted before use to the proper concentrations in the following manner.

For whole blood, 10 ml. of the sodium standard plus 10 ml. of the potassium standard are diluted to the mark in a 100 ml. volumetric flask.

For plasma, the standard for whole blood is used for the sodium determination. For the potassium determination 5 ml. of the potassium standard plus 30 ml. of the sodium standard are diluted to the mark in a 100 ml. volumetric flask.

For red blood cells, the standard for whole blood is used for potassium. For sodium, 5 ml. of the sodium standard plus 50 ml. of the potassium standard are diluted to the mark in a 100 ml. volumetric flask.

For urine, any one of the standards may be employed (depending on how dilute the urine is) with the provision that the standard must contain a higher concentration of the element being determined.

Preparation of Samples

Plasma—2 ml. of plasma are diluted with 20 ml. of distilled water and the proteins precipitated by slow addition of 3 ml. of 20 per cent trichloroacetic acid. The precipitate is removed by centrifugation and a portion of the supernatant (20 ml.) is run through the flame photometer to determine the K concentration. Since normal plasma contains between 315 and 345 mg. per cent of Na and but 16 to 22 mg. per cent of K, and since the presence of large amounts of Na affect the K readings (but not vice versa), calibration of the instrument, as noted above, *must* be based on solutions containing similar relative proportions of these ions. In practice, the calibration curve used for K in plasma is constructed from readings on solutions containing between 0 and 300 p.p.m. of Na and 0 and 50 p.p.m. of K (Fig. 3). At the dilution of plasma used, the scale readings lie between 20 and 35, which represents between 13 and 20 p.p.m. of K.

5 ml. of the supernatant are further diluted to 25 ml. with distilled water and the determination of Na is made on the resulting solution. The Na determination in plasma is based on a calibration curve obtained on solutions containing from 0 to 100 p.p.m. of Na and 0 to 100 p.p.m. of K. In the dilution used, the scale readings for plasma Na lie between 57 and 70, which corresponds to concentrations of from 52 to 62 p.p.m. of Na in the diluted unknown.

Whole Blood—2 ml. of heparinized blood are diluted with 20 ml. of distilled water with a consequent laking of the red blood cells. The

protein is precipitated by the slow addition of 5 ml. of 20 per cent trichloro-acetic acid and the precipitate is removed by centrifugation. 10 ml. of the supernatant are diluted to 50 ml. with distilled water and both Na and K are determined on the resulting solution. Since whole blood contains approximately equal concentrations of Na and K, the calibration curve in this case is based on solutions containing between 0 and 100 p.p.m. of Na and 0 and 100 p.p.m. of K. In the dilution of whole blood used, scale readings for Na lie between 20 and 45, corresponding to between 15 and 40 p.p.m. of Na, while the scale readings for K lie between 30 and 40, corresponding to between 20 and 35 p.p.m. of K in the diluted unknown.
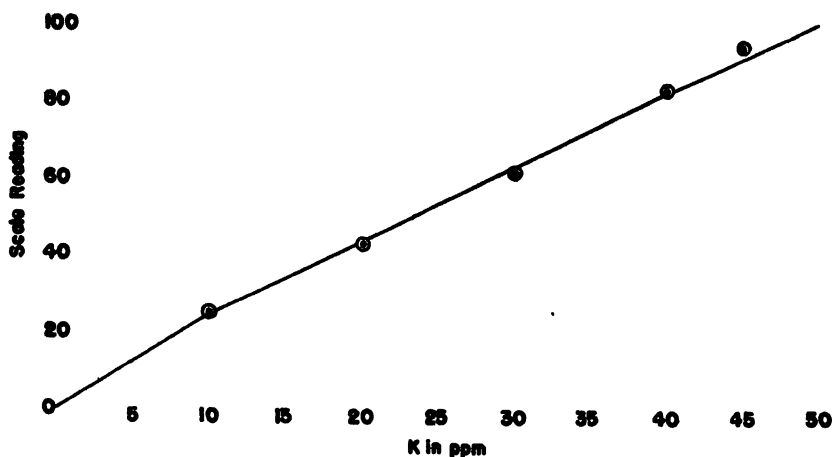
Fig. 3 Calibration chart for K (0 to 50 p.p.m.) in the presence of 0 to 300 p p.m. of Na.

Red Blood Cells—Erythrocytes are separated from plasma by repeated centrifugation at 3000 R.P.M. for a total of 1 hour. The plasma and the top layer of red cells are removed by a suction pipette and 1 ml. of red cells is pipetted from the bottom of the centrifuge tube and is diluted and laked with 20 ml. of distilled water. The protein is precipitated by the slow addition of 3 ml. of 20 per cent trichloroacetic acid. The pre-cipitate is removed by centrifugation and Na is determined on the resulting supernatant. By this method normal human red cells appear to contain less than 20 mg. per cent of Na.

10 ml. of the supernatant are further diluted to 50 ml. with distilled water and K is determined by means of a calibration curve based on solutions containing between 0 and 100 p.p.m. of Na and 0 and 100 p.p.m. of K. Scale readings, with such a dilution, lie between 35 and 40, cor-responding to between 30 and 35 p.p.m. of K.

Urine—1 to 3 ml. of urine is diluted to 100 ml. with distilled water and the resultant solution is used directly to determine both Na and K. The calibration charts used for these determinations depend entirely upon how concentrated the urine is. Trial runs are made in order to ascertain whether the scale is to be set at 100 p.p.m. or higher.

With the methods of sample preparation described above, values for Na and K in whole blood, plasma, red blood cells, and urine have been obtained on 60 human subjects. The average values and the ranges are presented in Table II. It will be seen that the average values obtained by this method agree quite well with the usually accepted values established by the more laborious and tedious chemical methods (9–12).

TABLE II

Comparison of Average and Range Data of Flame Photometric and Chemical Methods for Na and K Determination in Human Body Fluids

		Flame photometer, average*	Flame photometer, range	Average previously reported†	Range previously reported†
Whole blood	Na	218	190–257	200	170–225
	K	222	145–257	200	150–250
Plasma	Na	352	323–366	340	315–345
	K	20	14– 28	20	16– 22
Red blood cells	Na	14	Trace to 31	0	
	K	437	425–444	420	
Urine	Na	220	80–385		
	K	197	105–465		

* Average of determinations on thirty human subjects.
† See references (9–12).

DISCUSSION

The methods described here should be valuable not only because they give results as accurate as do the usual chemical methods but also because they materially reduce the time necessary to determine both Na and K. Perhaps the greatest advantage of flame photometry lies in the fact that it is not necessary to separate Na from K in order to determine either of them by this method.

However, it must be pointed out that considerable care is required in making standards, in calibrating the instrument and rechecking the calibrations during the runs, in preparation of the unknown solutions, and in operation of the instrument in order that one may rely on the results.

Numerous other methods of preparing biological fluids for flame photometric determination of Na and K were tried unsuccessfully. Wet ashing methods with various combinations of sulfuric and nitric acids, sulfuric and perchloric acids, and sulfuric acid and hydrogen peroxide were attempted. Each of these methods depends upon the presence of some sulfuric acid which must be neutralized (with NH_4OH) before the solution can be run through the flame photometer. None of these methods has been successful, since, apparently, the presence of large amounts of salt (in this case $(NH_4)_2SO_4$) causes a depression of both the Na and K curves (2).

Dry ashing methods are likewise unsuccessful because splattering and creeping of the solutions occur, the masses of residue left after ashing small blood samples are extremely minute and consequently difficult to handle quantitatively, and, finally, in the case of whole blood or red cells, complex iron compounds are formed which possess extremely low solubilities.

In flame photometric determination it is not necessary to remove all organic material before the solution is run through the instrument. It *is* advantageous to be rid of most of the protein, which may clot or precipitate and clog the aspirator tubing. Such a degree of protein removal is readily obtained with 20 per cent trichloroacetic acid and, since the samples are so highly diluted, no serious coprecipitation of ions with the protein is encountered.

SUMMARY

1. Rapid procedures for flame photometric analysis of Na and K in blood, plasma, red blood cells, and urine are described.

2. The accuracy of these more rapid methods approximates that of the more laborious chemical methods usually employed in Na and K analysis.

3. Average and range data from 60 control determinations on human subjects are presented and compared with data previously reported.

4. Some of the precautions to be taken in the operation and calibration of the flame photometer are discussed.

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PROPERTIES OF TOMATO PECTASE*

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Pectase, the enzyme catalyzing the deesterification of pectin, has been used as a catalyst in the preparation of low ester pectins (1–3), partially deesterified products showing promise of commercial importance.

The literature on pectase prior to 1936 has been reviewed by Kertesz (4) who points out that many of these early studies are of doubtful value because the data were based upon gel formation, which is not a true measure of the reaction involved. The direct titration of liberated carboxyl groups is a valid measure of pectase action and has been used in recent studies on pectases derived from tobacco (4), tomatoes (5–8), alfalfa (9), and citrus peel (2, 10). These studies provide information regarding the general nature of the enzyme and indicate the non-identity of pectases derived from different sources.

The recent reports by Lineweaver and Ballou (9, 11) that pectase activity is influenced by cations has both theoretical and practical significance. It explains the earlier observation by Hills *et al.* (1) that tomato pectase activity was increased by the addition of sodium oxalate. The application of these findings to the measurement and utilization of pectase is obvious.

Only a few published studies are available regarding the properties of tomato pectase. Kertesz (5) studied the effects of substrate and enzyme concentrations on the determination of pectase activity. The optimum pH was not determined, but the data indicate that it is 6.2 or above. A subsequent study (6) showed that ripe tomatoes contain more pectase than immature fruits. Pectinase, the enzyme hydrolyzing the glycosidic linkages of the pectin molecule, was detected in ripe tomatoes but not in green tomatoes. Studies on the pasteurization of tomato juice (7) showed that tomato pectase was completely destroyed by heating to 80° for 45 seconds or to 70° for 2.5 minutes. The time required for complete inactivation was affected by the pH of the mixture, being less for juices acidified below the natural pH (about 4.0).

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The preparation of tomato pectase was studied by Willaman and Hills (8). They observed that the enzyme was adsorbed on the pulp solids at the natural pH of the tissue but was desorbed by the addition of alkali. Thus above pH 5.0, clear filtrates free of suspended solids and rich in pectase were readily prepared.

Tomato pectase was studied because it is a cheap and convenient catalyst for the deesterification of pectin. The present paper presents further data on the preparation and properties of tomato pectase.

Materials and Methods

Preparation of Pectase—The pectase solution used throughout this study was a clear yellow filtrate prepared from firm ripe tomatoes by the modification previously described (12) of the Willaman and Hills (8) method. Briefly, the method consisted in grinding firm ripe tomatoes to a pulp, adjusting to pH 7.5 by the addition of 5 N NaOH, and filtering. The extract was stored under xylene at 0°.

Preparation of Pectin—The purified apple pectin used as substrate in most of these studies was prepared from dried apple pomace by extraction with 14 parts by weight of water at 80° for 70 minutes at pH 2.5, oxalic acid being used as the acidulant. The extract was pressed out, treated with diastase to remove starch, filtered, and concentrated *in vacuo* to about 2 per cent pectin. The pectin was purified by the following modification of the method of Olsen *et al.* (13). 3 per cent of concentrated hydrochloric acid by volume was added to the pectin concentrate; the mixture was stirred and allowed to stand for 10 minutes, then precipitated by stirring into 2 volumes of 80 per cent alcohol. The precipitate was washed on a filter until free of chlorides, pressed, dried overnight at 60°, and ground to pass a 60 mesh screen. 4.9 per cent moisture, 0.39 per cent ash (moisture-free basis), and 10.24 per cent CH_3O (Zeisel), were obtained on the alcohol-free sample (12). The pectin was further characterized (14) as being 76.4 per cent methyl-esterified and containing 80.7 per cent methyl galacturonide.

Method of Pectase Determination—The method used to measure pectase activity was based upon the Kertesz (5) titration technique, in which the carboxyl groups liberated by pectase hydrolysis are titrated with standard alkali. The original method was modified by the use of a continuous electrometric titration at constant pH (1, 3) and by the addition of electrolyte as recommended by Lineweaver and Ballou (9, 11). The optimum conditions of pH and electrolyte concentration were determined and used in all measurements unless otherwise specified. This method is similar to the procedure used by Lineweaver and coworkers (9, 10).

The standard procedure was to measure into a 600 ml. beaker 200 ml.

of a 1 per cent solution of purified apple pectin, 12.5 ml. of 2 M sodium chloride, 5 ml. of 0.2 M sodium oxalate, and sufficient distilled water to give a final volume (after the addition of the enzyme) of 500 ml. The substrate thus contained 0.4 per cent pectin, 0.05 M sodium chloride, and 0.002 M sodium oxalate.

The reaction mixture was adjusted to 30° ± 0.1° and maintained at that temperature in a water bath. It was stirred by a small electrical stirrer, and the pH was measured by a Beckman pH meter equipped with extension leads. The enzyme solution (usually 5 ml.) was added and the mixture, while being stirred, was quickly titrated to pH 7.5 or slightly above. The amount of deesterification which occurred during the addition of pectase and the neutralization of the reaction mixture was usually less than 0.2 ml. of 0.5 N NaOH, and therefore could be ignored.

The pH meter was set at pH 7.50 (corrected for the pH drift caused by stirring (12)), and observations of time and alkali consumption were begun at the instant the galvanometer needle of the pH meter crossed the zero· mark on the dial. This provided a very sensitive means for measuring the start and the end of the reaction. The pH was maintained constant within 0.1 pH unit by the dropwise addition of 0.5 N NaOH. Ordinarily the reaction was allowed to proceed until 3 or 4 ml. of 0.5 N NaOH had been consumed. By measuring the elapsed time to the closest second and applying a temperature correction (0.4 per cent per 0.1° deviation from 30.0°), the precision of the method, as determined by the average deviation of a number of duplicate determinations, was 0.02 ml.

For measurements made between pH 7.5 and 5.0, the titration values were corrected for the incomplete neutralization of the substrate. Below pH 5.0, it was necessary to use a method based upon the difference in titration (to pH 7.5) of two mixtures, one at constant pH for a given time and the other titrated immediately. This required a carefully standardized titration procedure, especially with respect to the time consumed in the final neutralization.

Discussion of Method—The choice of a method for enzyme assay and of units for expressing activity depends upon an understanding of the nature of the reaction involved. Published studies on pectase disagree as to whether the enzyme deesterification of pectin is a first order or a zero order reaction. Kertesz (4) reported that tobacco pectase followed a first order reaction, whereas Lineweaver and Ballou (9) found that the reactions catalyzed by alfalfa and pea vine pectases approximated a zero order reaction over the initial 50 per cent hydrolysis. More recently Speiser *et al.* (15) studied the kinetics of tomato pectase action on pectin but failed to prove whether the deesterification is a zero or first order reaction.

Fig. 1 shows that the course of tomato pectase deesterification is zero order for the initial 40 or 50 per cent hydrolysis but thereafter deviates from a zero order reaction. The deesterification does not correspond to a first order reaction over any portion of the curve. These conclusions
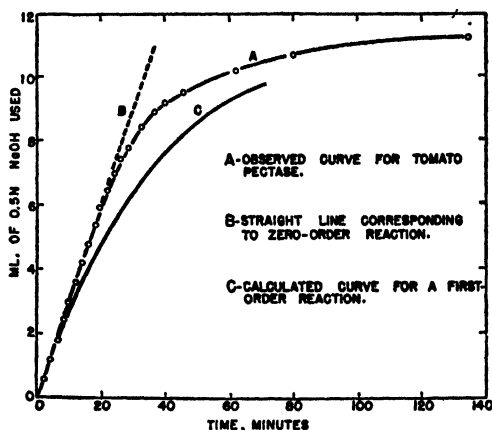
FIG. 1. Typical enzyme deesterification curve

TABLE I

Comparison of Zero and First Order Constants for Pectase Deesterification of Pectin (Standard Substrate Plus 5 Ml. of Pectase)

Fraction	Zero order, $K = \dfrac{x}{t}$ *	First order, $K = \dfrac{2.303}{t} \log_{10} \dfrac{a}{a-x}$
per cent		
0–10	0.150	0.0272
10–20	0.152	0.0317
20–30	0.150	0.0354
30–40	0.150	0.0414
40–50	0.140	0.0458
50–60	0.129	0.0512
60–70	0.099	0.0508
70–80	0.053	0.0377
80–90	0.021	0.0390
90–95	0.006	0.0205

* K is expressed as milliequivalents $\times$ min.$^{-1}$.

were confirmed by calculating the zero and first order constants for 10 per cent increments of the deesterification (Table I). From these data it is clear that the initial reaction is zero order instead of first order.

The present procedure, based upon the determination of pectase activity

within the region of the zero order reaction, satisfies the test for a valid method of measurement (16) in that the rate of activity is proportional to enzyme concentration over a wide range of added enzyme, as is shown later.

Pectase activity is expressed as the zero order velocity constant, where K is in units of milliequivalents of bonds hydrolyzed per minute. This unit is similar to that recommended by Lineweaver and Ballou (9). The ratio between the present unit and that proposed by Kertesz (5) is theorectically 1:930. However, since the Kertesz method does not measure pectase activity at the optimum pH or optimum electrolyte concentration, the ratio of the activities by the two methods may range from 1:300 to 1:800, depending upon the ion content of the pectin used as substrate.
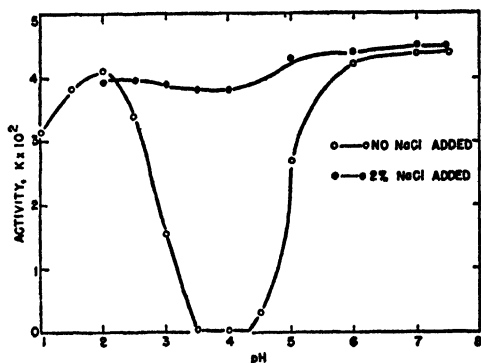
FIG. 2. Desorption of pectase from tomato pulp

Results

Desorption of Pectase from Tomato Pulp—The previously reported study of the desorption of pectase from tomato pulp by dilute alkali (8) has been extended to include the effects of acids and neutral electrolytes. Filtrates were prepared from 50 ml. portions of raw tomato pulp adjusted to the desired pH by the addition of either concentrated hydrochloric acid or 5 N NaOH. At pH 2.0 and below, the pectase activity of the filtrates was obtained by measurements made 30 and 60 minutes after the addition of acid, with extrapolation to zero time.

Fig. 2 shows that without added sodium chloride there is a region of minimum desorption near pH 4.0 with maxima at pH 2.0 and 7.5. The addition of 2 per cent sodium chloride was effective over the entire pH range investigated. It was also observed that at pH 6.0 or above the presence of 2 to 5 per cent of added sodium chloride caused a 10-fold

increase in the rate of filtration of the pectase extract through medium grade filter paper.

The use of 2 per cent sodium chloride at pH 6.0 or acidification to pH 2.0 without added salt would be suitable procedures for the large scale preparation of tomato pectase extracts. Extracts prepared at pH 2.0 should be adjusted to pH 4.0 prior to storage.

For the purpose of the present study, desorption at pH 7.5 without added salts was used in order to obtain an extract of low ionic concentration. The ion concentration of the extract was 0.11 M, determined by conductivity and calculated as KCl.

Stability of Tomato Pectase Extracts—In order to select the optimum conditions for storage and to anticipate the rate of deterioration, tomato pectase extracts were stored under various conditions of temperature and pH.

TABLE II

Effect of pH on Stability of Tomato Pectase Extracts Stored at 0° and 23°

pH	Rate of loss of activity	
	0°	23°
	per cent per mo.	*per cent per day*
7.5	2.6	1.42
5.0		0.55
4.0	1.0	0.42
3.0		0.88
2.0	12.7	20.0

50 ml. aliquots of tomato pectase extract were adjusted to the desired pH and stored at either 0° or at room temperature (23°). A few drops of xylene were added as a preservative. Pectase activity was determined at the start and at intervals during storage.

Table II shows the rate of deterioration of extracts stored at various pH values. The enzyme appears to be most stable at pH 4.0, the natural pH of tomato tissue. The addition of 2 per cent sodium chloride did not affect the stability of the enzyme at pH 4.0 and above but had a deleterious effect at a lower pH.

These data indicate that tomato pectase extracts may be stored for several days at room temperature or several months at 0° without appreciable loss. The pectase derived from tomatoes appears to be much more stable than that derived from citrus peel (10) or from alfalfa (9).

Effect of Enzyme Concentration—The activity per ml. of pectase extract, with the standard substrate, was observed to be constant over a wide range

of enzyme concentration (Table III).   It was not possible to make accurate measurements with amounts of pectase extract greater than 20 ml.

The constancy of the pectase activity per ml. of added enzyme is important in the determination of pectase activity and appears to hold for all concentrations commonly encountered.   It also indicates the validity of the method used for pectase determinations.

TABLE III

Effect of Enzyme Concentration on Pectase Activity

Tomato pectase added	Activity
ml	*m eq. per ml. per min.*
20 0	0.0285
10.0	0.0286
5.0	0.0287
2.0	0.0286
0.5	0.0284
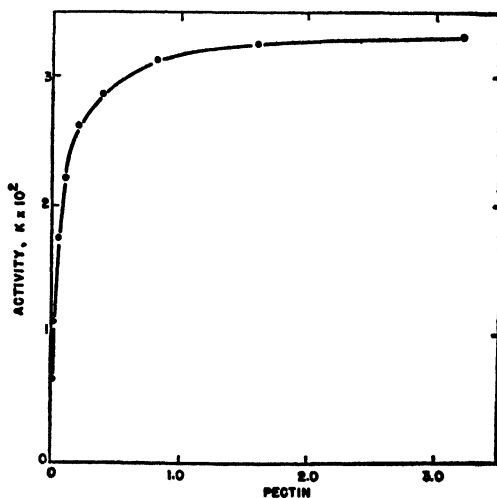
FIG 3. Pectase activity as a function of substrate concentration

Effect of Substrate Concentration—The dependence of pectase activity on pectin concentration was determined by measuring the rate of deesterification of substrates containing amounts of pectin ranging from 0.01 to 3.2 per cent   In all cases, readings were taken before 40 per cent of the available methyl groups had been hydrolyzed.

The data (Fig. 3) indicate that pectase activity increases with pectin concentration, practically attaining a plateau above 1.6 per cent pectin.

At 0.4 per cent pectin, the concentration used for pectase assay, the activity was 85 per cent of that at 3.2 per cent pectin.

It was of interest, both from a practical and a theoretical standpoint, to determine the concentration of pectin which will give the maximum rate of deesterification per unit of enzyme. This was done by applying the Lineweaver and Burk (17) form of the Michaelis-Menten (18) equation

$$\frac{(S)}{v} = \frac{(S)}{V} + \frac{K_s}{V}$$

where (S) is the substrate concentration, v the observed velocity at a given substrate concentration, V the maximum velocity, and K_s the Michaelis-Menten constant. By plotting $(S)/v$ against (S), it was possible to determine V and K_s from the slope $1/V$ and the intercept K_s/V.

The maximum velocity was calculated to be 0.0334 (milliequivalent per ml. per minute), corresponding to about 3.2 per cent pectin concentration. The value for the Michaelis-Menten constant, 0.041 per cent, may be compared with the values 0.04 per cent for alfalfa pectase (9) and 0.08 per cent for citrus peel pectase (10).

It should be pointed out that the Michaelis-Menten equation applies only to the initial 40 per cent of the pectase deesterification curve. Beyond this point in the deesterification reaction there was a marked curvature in the plot of $(S)/v$ versus (S). This is not entirely unexpected because, as the deesterification reaction proceeds, the substrate changes both quantitatively (with respect to methyl ester content) and qualitatively (with respect to magnitude of the negative charge). The Lineweaver-Ballou hypothesis (9) concerning the formation of an inactive ionic complex between pectinic acid and pectase offers a possible explanation of this behavior.

Effect of Electrolyte Concentration—The recent studies by Lineweaver and Ballou (9) and by MacDonnell *et al.* (10) indicate the dependence of pectase action on electrolyte (cation) concentration. The concentration required for maximum pectase activity is a function of the pH and of the valency of the cation.

The fact that alfalfa and citrus peel pectases show differences in behavior toward cation concentrations indicates that this effect is also a function of the source of the enzyme.

In the present study the effect of sodium chloride concentration on tomato pectase activity was determined at pH 7.5, 6.0, 5.0, and 4.0. The results (Fig. 4) show that both the optimum salt concentration and the maximum activity are affected by pH. The optimum salt concentrations at pH 7.5, 6.0, 5.0, and 4.0 were 0.05, 0.08, 0.15, and 0.30 M Na ion, respectively. These values are nearly identical with those reported for alfalfa pectase (9) but are appreciably smaller than those for citrus peel pectase (10).

It was impossible to obtain enzyme-substrate mixtures entirely free of cations because small amounts were contributed by the alkali used for the original neutralization (1.5 milliequivalents), by the ash of the pectin substrate (0.5 milliequivalent), and by the pectase solution (0.5 milliequivalent). When dialyzed pectase was used without added electrolyte, the activities at pH 7.5 and 6.0 were 0.0054 and 0.00026 milliequivalent per ml. per minute, or 18 and 1 per cent of the respective maxima at optimum salt concentrations.

Tomato pectase activities at pH 7.5 in the presence of 0.05 M sodium acetate, sodium chloride, or potassium chloride were in the ratio of 100:97:97, indicating that the anion may have a slight effect and that sodium and potassium are equivalent.
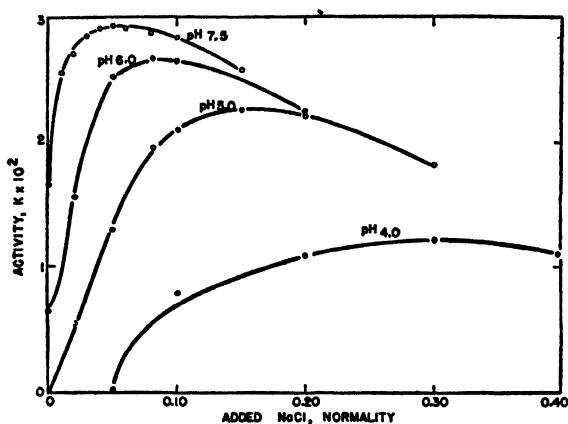
Fig. 4. Effect of NaCl on pectase activity at various pH levels

Effect of pH on Pectase Activity—The optimum pH for tomato pectase was determined in the presence of 0.05 M sodium chloride. Measurements between pH 7.5 and 5.0 were made by direct titration at constant pH, the results being corrected for incomplete neutralization of the substrate. At a lower pH, the results were obtained by titrating (to pH 7.5) two mixtures that had been allowed to react for different times, as described previously. Above pH 7.0, it was necessary to correct for the amount of deesterification caused by alkali. In Fig. 5, Curve A-B represents the observed rate of deesterification produced by enzyme and alkali together. Curve C shows the rate for alkali alone, and Curve A-A' for the enzyme alone.

Tomato pectase in the presence of 0.05 M sodium chloride shows a maximum activity at pH 7.5 with a fairly broad optimum region extending from about pH 6.0 to 9.0. The data presented previously (Fig. 4) show that the optimum salt concentration increases at a lower pH. Thus 0.05 M NaCl, which is optimum for pH 7.5, is suboptimum for pH 6.0 or 5.0. From a

practical point of view it is desirable to show the maximum activity and required salt concentration in the range of pH below 7.5. These results, plotted in Curve D, Fig. 5, show that at a lower pH a material increase in pectase activity may be realized by increasing the salt concentration.

Temperature Coefficient—The influence of temperature on the rate of pectase action was studied under conditions nearly identical with those recommended for the large scale preparation of low ester pectins (19). Rate measurements were made at pH 6.5 and at temperatures ranging from 10–65° (Fig. 6). Readings were taken over a period of 30 minutes, although
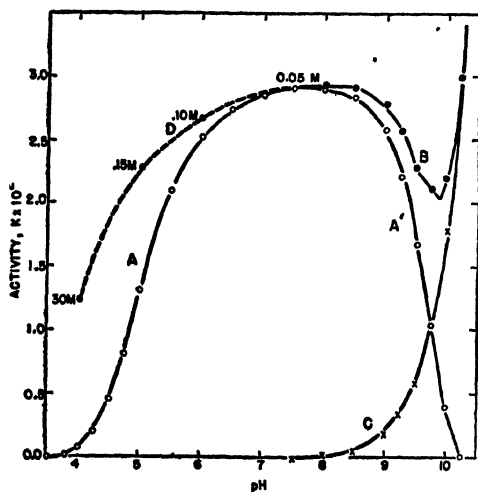
FIG. 5   FIG. 6

FIG. 5. Activity-pH curve for tomato pectase. Curve A-B, observed deesterification; Curve C, deesterification by alkali; A-A', corrected curve for pectase alone; Curve D, activity, pH 4.0 to 7.5, with optimum NaCl concentration for each pH.

FIG. 6. Effect of temperature on rate of pectase action at pH 6.5. Substrate, 0 8 per cent pectin, 0.05 N NaCl, 0.002 N Na oxalate. 2 0 ml. of tomato pectase extract used.
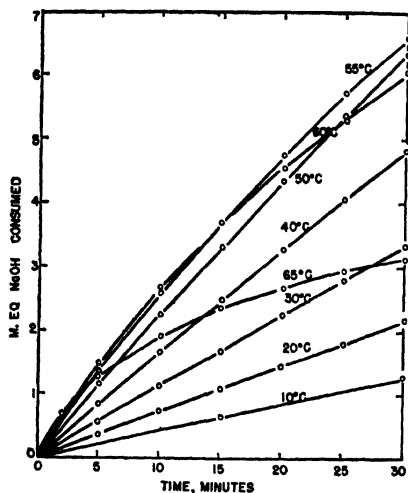
in some instances the reaction was allowed to continue for 1 hour. In all cases, the readings were confined to the initial 40 per cent portion of the deesterification reaction. Thus any deviation from linearity in these data is an indication of enzyme denaturation.

The "optimum temperature" for pectase action decreased with time, because at increased temperatures denaturation of the enzyme became an important factor. For a 2 minute reaction time the optimum temperature was 65°, for a 30 minute reaction it was 55° and for 1 hour (data not shown) it was 50°.

The linearity of the curves for 10°, 20°, 30°, and 40° indicates that denaturation of the enzyme was negligible under these conditions.

The increase in rate of an enzyme reaction with increase in temperature may be conveniently expressed as the Q_{10} value, the ratio of K_{t+10}/K_t. The Q_{10} values for tomato pectase shown in Table IV are much lower than the range of 2 to 3 predicted by the van't Hoff rule for catalyzed reactions in aqueous solution, and lower than is generally observed for other enzymes (20).

TABLE IV

Temperature Coefficient of Tomato Pectase Deesterification of Pectin

0.8 per cent pectin substrate, 0.05 M NaCl, 0.002 M Na oxalate, pH 6.5.

Temperature interval	Temperature coefficient, Q_{10}
°C.	
10-20	1.60
20-30	1.52
30-40	1.44
40-50	1.32
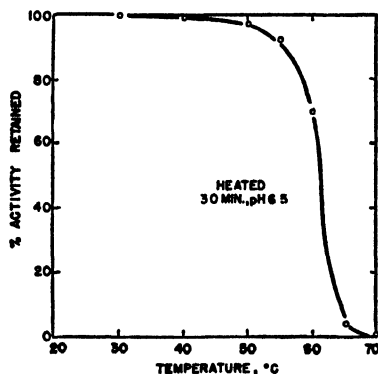
FIG. 7. Inactivation of pectase by heat

A value of 7280 calories for the activation energy was determined from the slope of $1/T$ *versus* log K (zero order). Initial reaction velocities were used in the calculations and the data were confined to the temperature range of 10-40° at which heat inactivation of the enzyme is not apparent. This is higher than the value 5790 calories previously reported by Speiser *et al.* (15), who used slightly different experimental conditions and based their results on the analysis of the isolated reaction product. A value of 6000 calories has been reported for citrus pectase (2).

Heat Inactivation—Fig. 7 shows the loss in activity caused by heating a

tomato pectase solution for 30 minutes at pH 6.5. The temperature (62°) required for 50 per cent inactivation is about 12° higher than that required for citrus peel pectase (10). This agrees with the general observation that tomato pectase is more stable in aqueous solution than is alfalfa or citrus peel pectase.

DISCUSSION

The differences in properties between tomato pectase and other plant pectases, while chiefly quantitative, are distinct enough to indicate the non-identity of pectases from different sources. The optimum pH and salt concentration for tomato pectase are pH 7.5 and 0.05 M NaCl. These values may be compared with pH 7.5 and 0.15 M NaCl for orange peel pectase (10) and pH 6.5 (salt concentration not determined) for tobacco pectase (4). Alfalfa pectase (10) is unique in that it shows maximum activity over the entire range from pH 5.6 (0.15 M NaCl) to pH 8.0 (0.02 M NaCl). Tomato pectase extracts are much more stable at 0° or room temperature than are extracts derived from alfalfa or citrus peel. Tomato and citrus pectases show differences in the effects of pH and salts on stability. The temperature required for heat inactivation of tomato pectase is about 12° higher than for citrus peel pectase.

On a fresh weight basis, the pectase activity of ripe tomatoes is about the same as that of fresh citrus peel and about 5 times that of fresh alfalfa (10). Crude pectase extracts prepared from tomatoes are about as active as the whole tissue. The efficient extraction of the enzyme from citrus peel or alfalfa requires the addition of several volumes of solvent, and the resulting extracts would be much less active than tomato extracts. Crude tomato pectase extracts may be prepared without added solvent and are sufficiently stable to permit their storage without further purification.

The present study indicates various means for improving the efficiency of pectase action. The activity per unit volume of pectase may be increased by deesterifying at a pH near 7.5 and adding the optimum quantity of electrolyte. The fact that pectase activity increases with increased substrate concentration may be applied to commercial practice by concentrating the pectin extract before deesterification. The use of increased temperatures is an obvious method of increasing the efficiency of pectase action. For most purposes, however, this method encounters the limitations imposed by the susceptibility of the pectin substrate to heat degradation (19). A fuller discussion of the utilization of tomato pectase for the large scale preparation of low ester pectins is presented in a separate publication (19).

SUMMARY

1. The deesterification of pectin by tomato pectase follows a zero order (for the initial 40 per cent) rather than a first order reaction. This fact

- was utilized in determining reaction rates and in choosing units for expressing activity.

2. Tomato pectase was prepared from tomato pulp by alkaline desorption. The desorption may be accomplished also by acid or neutral electrolytes.

3. The relation of enzyme activity to substrate concentration conforms to the Michaelis-Menten theory. The K_s value of 0.046 per cent is comparable to that found for alfalfa pectase but differs from that for citrus pectase.

4. Tomato pectase has a broad optimum pH region, with a maximum at 7.5.

5. The addition of salts is necessary for maximum activity. The optimum salt concentration at pH 7.5 is 0.05 M NaCl but is greater at lower pH values.

6. The enzyme was most stable in aqueous solution at pH 4.0. At this pH and 0° it deteriorated about 1 per cent per month. A temperature of 70° was required for complete inactivation in 30 minutes at pH 6.5.

7. The temperature coefficient (Q_{10}) was 1.52 for the region of 20–30°. The Arrhenius energy of activation in the region from 10–40° was 7280 calories. Above 40° inactivation of the enzyme became apparent.

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SEPARATION AND CHARACTERIZATION OF SOME PENICILLINS BY THE METHOD OF COUNTER-CURRENT DISTRIBUTION

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The recent brief summary (1) of the results obtained by the large group of British and American chemists made known to the general scientific public for the first time that a number of antibiotics of the penicillin class had been isolated. At least four are known to be commonly produced by various strains of *Penicillium*. These in the past have been called penicillins G, F, X, and K by the American workers. All have the same structure with the exception of a side chain, which difference is indicated in the new nomenclature for penicillins.[1] The above penicillins are designated respectively benzylpenicillin, Δ^2-pentenylpenicillin, *p*-hydroxybenzylpenicillin, and *n*-heptylpenicillin.

That the individual penicillins exhibit different antibiotic potencies and pharmacological properties has now been amply shown by the thorough studies of Eagle and Musselman (3), of Coghill, Osterberg, and Hazel (4), and others (5). The necessity of using pure preparations of individual compounds for any fundamental clinical or pharmacological study poses a problem of fundamental nature for the chemist, that is not only to obtain the pure single antibiotic in sufficient quantity but also to supply convincing evidence that only a single species, or at least a single species in high percentage, is present in the sample to be studied.

For the particular case of the penicillins this is an especially difficult problem in view of the following properties. The penicillins are unstable acids which are difficult to study as such and to characterize by the usual determinations of melting point, rotation, refractive index, etc. Ultimate analysis is not alone sufficient to demonstrate homogeneity or the presence of a single species only. Crystalline derivatives which retain their biological activity appear to be restricted to salts of the acids with either inorganic or organic bases. These salts do not possess melting points suitable for precise characterization.

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[1] The nomenclature used for the penicillins in this article conforms with that proposed by the Editorial Committee of the forthcoming monograph on the chemistry of penicillin (see (2)).

From this array of unfavorable properties it is indeed apparent that the conventional methods of organic chemistry are scarcely adequate. Some new approach would thus be highly desirable.

The use of partition coefficients for such problems is an old procedure, though in the past methods for employing these perfectly reliable physical constants have been so lacking in precision and convenience that they have not found the wide usage in organic chemistry which they properly deserve. With respect to the penicillin field this approach has already been suggested by the published work of Bush, Goth, and Dickison (6) and in the unpublished work of groups in the United States and England on the use of the partition chromatogram of the Martin and Synge (7) type.

The procedure of counter-current distribution (8, 9) represents an attempt to make the partition coefficient a more usable physical constant in organic chemistry. It is based on a certain systematization of multiple extractions or transfers so that the mathematics of the binomial expansion may be directly applied in the interpretation of the results. It has been applied with success to homogeneity studies in the field of the synthetic antimalarials (9) and in the isolation of active principles from *Aspergillus ustus* (10), as well as in a considerable amount of unpublished work. It was also used in the isolation of synthetic benzylpenicillin by du Vigneaud, Carpenter, Holley, Livermore, and Rachele (2). A logical further step from the standpoint both of the development of the method and of penicillin chemistry is to attempt the application of the method to this interesting and important class of natural products. The present paper will record a number of our preliminary experiments with the penicillins.

Though it is entirely feasible by the procedures to be discussed to isolate pure crystalline individuals directly from the culture medium or from commercial samples of penicillin now on the market, this effort has been made largely unnecessary by the kind cooperation of a number of industrial producers[2] who have supplied us with crystalline preparations of the sodium salts of the four above-mentioned types of penicillins. This has greatly facilitated our study and our thanks are herewith given for their generosity.

As discussed in previous publications (9, 10), the most promising system, e.g. the combination of immiscible solvents, for the study of acids and bases is usually one in which a buffered aqueous solution is one of the two phases. Ethyl ether and 2 M phosphate buffer occurred to us at once as a promising combination. Thus far this combination has proved the best from the

analytical standpoint for the examination of benzylpenicillin and of Δ^2-pentenylpenicillin. For the examination of p-hydroxybenzylpenicillin, ether-3 M phosphate has been the best, and for the examination of n-heptylpenicillin, ether-1 M phosphate has been the system of choice. Other solvents such as chloroform and ethyl acetate have been used with partial success. Though ethyl acetate has admirable solvent properties, it has thus far with each of the four different penicillins always led to a certain percentage of inactivation.

In a previous publication (9) the desirability of working with a partition coefficient of about 1 for the characterization of a particular substance has been shown. The further desirability of choosing a buffer system and an organic solvent which would give the greatest shift of partition coefficient with the shift of pH also was shown. A preliminary study of these factors with a crystalline preparation of the sodium salt of benzylpenicillin showed that with ethyl ether and 2 M phosphate the partition coefficients were those shown in Fig. 1. The pH given is that recorded directly by use of the glass electrode at 25° and is relative only, rather than absolute, because of the high salt concentration. The partition coefficients were determined at 5° in a constant temperature room. The values given do not represent the highest precision but are sufficient for the purpose at hand, at least until the homogeneity of the preparation has been demonstrated. The concentration in each layer was determined spectrophotometrically by use of the Beckman quartz spectrophotometer.

Plotted in Fig. 1 are the values found in isopropyl ether-buffer for plasmochin, one of the synthetic antimalarial bases previously studied. It can be seen that the slope of the line is much greater for plasmochin, and therefore the specificity of the constant is greater. Ethyl acetate or chloroform with benzylpenicillin, Fig. 1, did not offer improvement. The prediction might be made from these data that difficulty would be experienced in attempting to spread the partition coefficients of two penicillins whose partition coefficients chanced to lie close together, by changing the solvents or conditions. In order to compensate for such an unfavorable property it is obvious that higher numbers of transfers would be required for a desired separation. Therefore, thus far those systems have been most studied which are the best suited for the greatest precision in the technical execution of the method. Ethyl ether is admirable from this standpoint.

In general use of the method of counter-current distribution it is desirable to be able to adjust the value of the partition coefficient at will. It is apparent from Fig. 1 that the buffered system will permit this in the case of the penicillins. At the same time conditions of relative stability for the penicillins may be obtained if the whole operation is performed in a constant temperature cold room at about 3–5°.
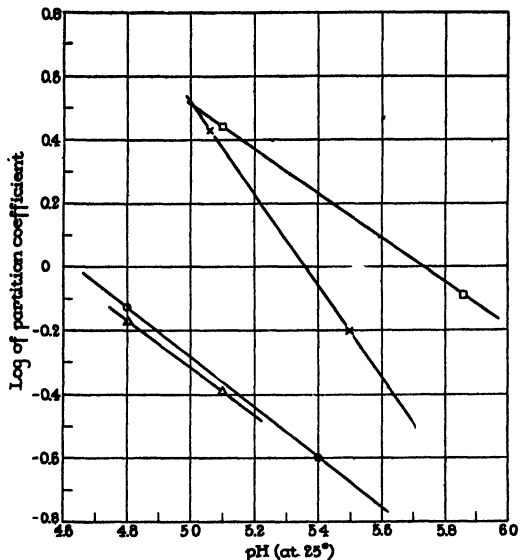
Fig. 1. Shift of partition coefficient with pH; ○ = benzylpenicillin in ether-2 M PO₄ buffer; □ = benzylpenicillin in ethyl acetate-2 M PO₄ buffer; △ = benzylpenicillin in 2 M PO₄ buffer-chloroform (1/logarithm of K is plotted), × = plasmochin in isopropyl ether-2 M PO₄ buffer (1/logarithm of K is plotted)
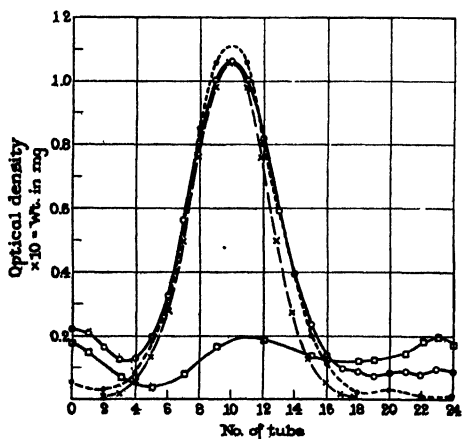
Fig. 2. Distribution pattern of crystalline benzylpenicillin; ○ = optical density at 260 mμ; □ = optical density at 320 mμ; ● = weight in mg.; × = calculated curve

Fig. 2 shows one of the earlier runs with a crystalline preparation of benzylpenicillin. It shows the maximum biological activity when com-

pared with the international standard and agrees in all other recorded properties.

The distribution was carried out as follows. 2 M buffer was prepared which contained 248 gm. of $NaH_2PO_4 \cdot H_2O$ (Merck) and 34.8 gm. of K_2HPO_4 (Merck) in a liter of solution. This buffer thus far has not caused trouble by crystallization in the cold room at 4°. It has shown repeatedly a pH of 4.8 when directly measured against the glass electrode at 25°.

250 cc. of buffer and 250 cc. of commercial absolute ethyl ether, previously redistilled, were shaken at 4° in a separatory funnel. The lower portion of each of the tubes of the apparatus was then filled with the buffer layer, 8 cc. with the present machine (9), and then an equal volume of the ether layer was added to each tube.

100 mg. of the penicillin were placed in a ground glass-stoppered tube of the same inside diameter and length as the tube of the machine. 8 cc. of the buffer layer were then added, and after solution 8 cc. of the ether layer were added. Equilibration was then accomplished as in the machine by inverting the glass tube and turning it back again to the original position 50 times. The degree of emulsification and the time for complete separation of the layers were noted. In the present case approximately 1 minute and 30 seconds were required for separation of the layers.

The contents of Tube 0 of the machine were then withdrawn and replaced by the contents of the glass tube. The layers were allowed to separate and then the upper part of the machine was rotated one stage so that the upper layer of Tube 0 was over the lower layer of Tube 1. The machine was equilibrated by inversion 50 times, the speed of inversion being controlled by observing the glass dummy attached to the outside, which was also filled with the same two layers. The layers were allowed to separate during a time lapse of 3 minutes before advancing to the next stage. When the upper layer of Tube 0 had advanced until it was over the lower layer of Tube 24, the contents of each tube were withdrawn into small separatory funnels numbered 0 to 24.

The optical density of each of the upper layers was then determined in the Beckman quartz spectrograph at 260 and at 320 mμ. This was done without dilution, and starting with Tube 0. After measurement, the contents of the cuvette were placed again in the separatory funnel by means of a pipette and the cuvette filled with the next layer, without rinsing.

After the first series of measurements 1 cc. of 8 M phosphoric acid was added to each of the separatory funnels and the funnel quickly shaken in order to transfer all the penicillin into the ether layer. Preliminary experiments had shown that this resulted in practically quantitative transference of the penicillin in the buffer layer to the ether layer. The optical density of each of the upper layers was again determined. From these two meas-

urements in each tube the partition coefficients can be calculated. In Fig. 2 the optical density of each tube is plotted against the consecutive number. of the tube.

The upper layer of each of the separatory funnels was then placed in a test-tube closed by a cork stopper covered with paper and stored overnight in a large thermos jar containing dry ice. Under these conditions nearly all the water is frozen out of the ether and with it any small amount of clinging buffer. No attempt was made to exclude CO_2 from the tubes, and its presence has thus far not appeared to interfere.

When the crystals of ice were found to be attached to the sides of the tube, it was quickly withdrawn from the jar and decanted into another fresh tube. If this did not give a clear cut separation, the ice was removed by use of an inverted filter. In the latter case the filter plug was a wad of cotton. Since the ice crystals were not routinely washed with fresh ether, there was a resultant loss of about 0.2 cc. of ether solution in each tube. This did not affect the quantitative interpretation, since the same volume was lost in each tube. Furthermore, there did not appear to be sufficient loss of the penicillin, other than that in the residual 0.2 cc. of ether, to cause an error in the quantitative determination. If the analysis were made with significantly larger amounts of penicillin, however, it is possible that appreciable losses would occur as a result of separation of the penicillin on the ice crystals.

The clear ether solution of every other tube (Tubes 0, 2, 4, etc.), starting with Tube 0, was then evaporated to dryness in a weighed Erlenmeyer flask, and the residue was dried at room temperature over calcium chloride at 0.2 mm. pressure. The weights of the penicillin in the tubes are shown in Fig. 2.

Although the residues in the tubes of the main band showed practically the theoretical biological activity against *Bacillus subtilis* when compared to the crystalline starting material, they were not completely soluble in ether, and it is likely that there was a small percentage of inactivation during the evaporation. However, when the small amount of insoluble amorphous material was removed by filtration, the crystalline triethylammonium salt of benzylpenicillin[3] could be prepared in nearly quantitative yield from the ether filtrate.

The triethylammonium salt could be more readily prepared directly from the dry ether layers of each tube. Addition of an excess of triethylamine either directly or in ether solution was followed by turbidity and almost immediately by quantitative crystallization in very fine needles.

[3] The use of triethylamine to prepare a crystalline salt of natural benzylpenicillin was first described by the investigators of the Heyden Chemical Corporation, May 22, 1944 (see (2)).

The crystals were collected in a centrifuge filter (11) and immediately dried under a vacuum at room temperature. The melting point of the crystals was not sharp, depended considerably on the rate of heating, and was found to be approximately 135–145° with previous sintering when determined on the micro hot stage melting point apparatus. The crystals showed full antibiotic activity against *Bacillus subtilis*. Material from Tubes 5 and 15 showed a slightly lower melting point, as well as activity, than those from Tube 10, but the difference did not appear to be large enough to be of much significance.

The curve of absorption at 320 mμ showed the presence of a small amount of another compound superimposed over the pattern of a penicillin. However, from a percentage standpoint the significant curve is always the weight curve, and this showed little weight to be present at certain tubes having the highest absorption at 320 mμ. The material responsible for this absorption probably did not represent more than a per cent or 2 of the total weight in the preparation.

From the standpoint of the investigation of homogeneity the significant feature of a determined curve is the closeness of its fit with a theoretical distribution (12) as well as the determination of partition coefficient of the material in each tube. When an attempt was made to fit a theoretical curve to the experimental absorption curve at 260 mμ the discrepancy shown in Fig. 2 was obtained. Further, the partition coefficients appeared to become consistently larger from left to right of the band. Had the attempt to fit a theoretical curve to the weight curve been made, a somewhat closer fit would have resulted. This will be shown in the next experiment. It would appear probable from Fig. 2 that the most accurate fitting of theoretical distributions as well as determination of partition coefficients cannot be done by means of absorption measurements in the demonstrated presence of a small percentage of a component absorbing more strongly at 320 mμ. However, the experiment as performed would appear sufficient only to demonstrate several per cent of inhomogeneity.

If the convenient use of ultraviolet absorption spectrum measurement cannot be regarded as reliable even for those penicillins which have a characteristic type of absorption, then the analysis must depend almost entirely on weight. In any case, weight represents the most fundamental and reliable method of determining the amount of material in a given solution. Experiments were, therefore, carried out to determine the precision with which penicillins could be determined by evaporation in ether solution and determination of the weight of the residue. For this purpose a series of Florence flasks were blown from soft glass vials. These flasks held a volume of approximately 12 cc. and weighed approximately 3 gm. each. The flasks were routinely weighed on a semimicro balance. They could be

rapidly weighed to ±0.02 mg. when counterbalanced with an empty flask of the same series to correct for any change in humidity.

Experiment showed that redistilled ether shaken at 5° with either 2 M buffer at pH of 4.80 or buffer acidified with 1 cc. of 8 M phosphoric acid showed no weighable residue when a 2 cc. volume was evaporated.[4] Experience also showed that in these flasks of very thin clear walls a residue greater than 0.02 mg. could be seen with the naked eye when the flasks were held against the light.

With the use of this technique a distribution was made on a different sample of several times recrystallized benzylpenicillin. The result is shown
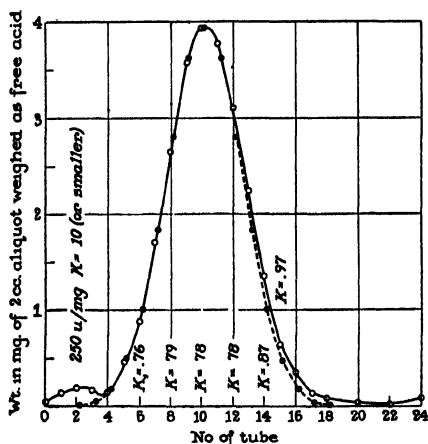
FIG. 3

FIG. 4

FIG. 3. Distribution pattern of four times recrystallized benzylpenicillin; O = experimental weight curve; ● = calculated curve.
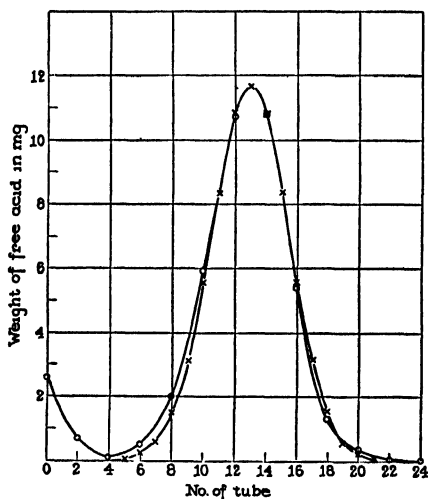
FIG. 4. Distribution pattern of Δ^2-pentenylpenicillin; O = experimental weight curve; × = calculated curve.

in Fig. 3. In this experiment the buffer of Tube 0 was adjusted to account for the sodium of the penicillin (1.6 cc. of 2 M NaH_2PO_4 and 6.4 cc. of the equilibrated buffer of pH 4.80). After the customary twenty-four transfers a final equilibration was made, and the material was withdrawn from the machine. The upper layers were separated from the lower and replaced by 8 cc. of fresh ether in each separatory funnel. 1 cc. of 8 M phosphoric acid was added and, after having been shaken, the ether layers were separated and labeled as the lower layers. A 2.00 cc. aliquot from

[4] It is thus evident that for analytical purposes only the step of freezing in dry ice is not necessary.

each of the layers of the tubes investigated was withdrawn and all evaporated at once under reduced pressure. From the weights obtained partition coefficients as well as total weights could be calculated. The results are shown in Fig. 3.

Tubes 0 to 3 contained an impurity which represented about 2 per cent of the starting material. This impurity showed a low antibiotic activity of about 250 units per mg. and probably contained a p-hydroxybenzyl type of penicillin. When an attempt was made to fit a theoretical curve on the main band, very good agreement was obtained until about Tube 13 was reached, but from this point on a small divergence was apparent. That this divergence was real was shown by the fact that the partition coefficients from Tubes 6 to 13 all agreed, but increased with Tubes 14 and 15.

A sample of the crystalline sodium salt of Δ^2-pentenylpenicillin was distributed in exactly the same system as was benzylpenicillin. Twenty-four transfers were applied (Fig. 4). The material occurring in Tubes 0 to 4 inclusive comprised about 6 per cent by weight of the total and showed no antibiotic activity. With the main band a small divergence from a theoretical distribution was apparent. This divergence occurred on the left side of the diagram, contrary to the experience with all the crystalline preparations of benzylpenicillin thus far examined. Comparison of the two distributions raises the suggestion that perhaps the crystalline benzylpenicillin preparations were contaminated with a few per cent of the Δ^2-pentenylpenicillin species, whereas the preparation of Δ^2-pentenylpenicillin contained a few per cent of the benzylpenicillin type. This could be perfectly possible without the fact being revealed by carbon and hydrogen analysis.

When the tubes from the peak of the band were treated with excess triethylamine and evaporated to dryness, a residue was obtained which, when moistened with acetone, and ether cautiously added, crystallized readily. The crystals were very well formed, stout, glistening columns which, however, did not show a sharp melting point. The substance melted in the range of 90–110°. The analytical data did not agree well with the calculated values, and perhaps a hydrate is indicated.

Calculation of the partition coefficient from the position of the maximum (8) gives the figure of 1.18 for Δ^2-pentenylpenicillin at 5° and in the system used. This is slightly higher than the partition coefficient 1.10 determined on the starting material. The discrepancy could easily be due to the 6 per cent of inactive material present in the original sample. The calculated value for benzylpenicillin (from the position of the maximum, Fig. 3) is 0.75. The ratio of the two partition coefficients, 1.57, gives a ready index of the difficulty of separating the two penicillins in the ether-phosphate system. Had the slope of the curve for benzylpenicillin in Fig. 1 been of the same order as that for plasmochin, then the separation of bands for

such closely related penicillins as benzylpenicillin and Δ^2-pentenylpenicillin probably could be achieved with fewer transfers. Although it would be highly desirable to find a system which would give a higher ratio, a number of experiments along this line have thus far been unsuccessful. Several other systems have instead brought the constants closer together; in the case of ethyl acetate for the organic solvent a slightly higher coefficient for benzylpenicillin than for Δ^2-pentenylpenicillin was even obtained. Further experiments will be made along this line when more Δ^2-pentenylpenicillin becomes available.
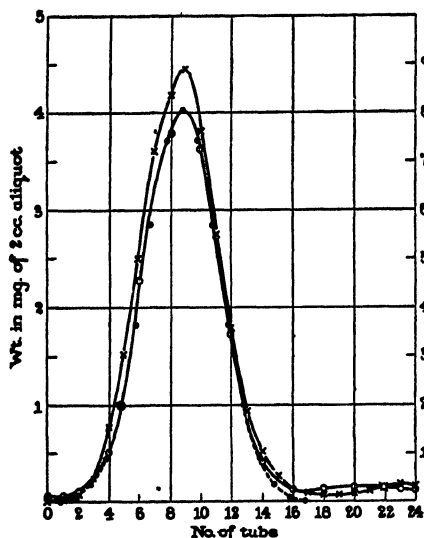
FIG. 5   FIG. 6

FIG. 5. Distribution pattern of p-hydroxybenzylpenicillin; $\bigcirc$ = experimental weight curve; $\times$ = optical density at 280 mμ; $\bullet$ = calculated curve.
FIG. 6. Distribution pattern of n-heptylpenicillin; $\bigcirc$ = experimental weight curve; $\bullet$ = calculated curve.
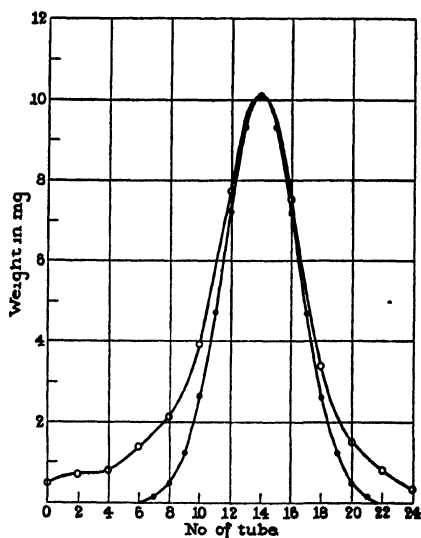
A sample of the crystalline sodium salt of p-hydroxybenzylpenicillin was studied. Preliminary investigation of the partition coefficient of this penicillin in the system used for the runs thus far shown revealed a value in the neighborhood of 0.1. Since this figure is not the optimum constant for obtaining the maximum information concerning homogeneity in the smallest number of transfers, a more concentrated buffer was used to raise the value of the constant.

3 M buffer was prepared which contained 372 gm. of $NaH_2PO_4 \cdot H_2O$ and 52.2 gm. of K_2HPO_4 in a liter of solution. This buffer has not thus far given trouble by crystallization during a run at 5°.

A routine twenty-four transfer distribution with 100 mg. of material gave the curve shown in Fig. 5. In this distribution the weight of material in each tube was determined by evaporating a 2 cc. aliquot, and the curve was also determined by measuring the extinction at 280 mμ. Aside from a few per cent of extraneous material occurring in Tubes 16 to 24, a slight divergence from the theoretical distribution occurred at Tube 6. The entire extinction curve, however, was somewhat displaced toward the left. Such a distortion could be caused by a small amount of impurity occurring in a band with maximum at 6 and having a higher extinction coefficient than p-hydroxybenzylpenicillin at 280 mμ.

The remainder of the material in the ether of Tubes 8 to 12 inclusive was treated with triethylamine. An amorphous precipitate was formed immediately. The solvent was quickly evaporated *in vacuo* from the precipitate, and the residue was taken up in a small volume of acetone. Cautious addition of a little ether and scratching induced crystallization. After the mixture had cooled overnight, the thin leaves were collected by means of a centrifuge filter. This yielded 41.7 mg. of material with the melting point of 126–136°.

$C_{22}H_{32}O_5N_2S$.   Calculated, C 58.51, H 7.38; found, C 58.21, H 7.21

A sample of the crystalline sodium salt of n-heptylpenicillin (penicillin K) was studied. Neither of the buffers used for the other penicillins was suited for the most precise characterization or investigation of homogeneity of this penicillin, since both gave partition coefficients in too high a range. 1 M buffer at pH 5.50 was found to be suitable. This buffer was prepared to contain 110 gm. of $NaH_2PO_4 \cdot H_2O$ and 34.8 gm. of K_2HPO_4 in a liter of solution.

A routine twenty-four transfer distribution with 100 mg. of the material gave the curve shown in Fig. 6. The first transfer gave an emulsion which did not separate well, and the layers were separated by centrifuging the glass tube. Four transfers were done in individual glass tubes, and at this point the layers separated satisfactorily. All were transferred to the machine after the pure solvent in the corresponding tubes had been withdrawn, and the distribution was continued in the machine. A lapse of 5 minutes time was allowed for separation of the layers. The amount present in a given tube was determined by evaporation to dryness after freezing out the water in dry ice.

Tubes 13 and 15 were treated with excess triethylamine. No precipitate occurred. On standing and scratching, crystals gradually formed, and an almost quantitative yield of well formed rods were obtained. This material appeared to give the best melting point of a triethylammonium salt of any penicillin thus far studied. It melted at 110–113°, the values depending

somewhat on the rate of heating.   It was dried for analysis at room temperature at 0.2 mm. pressure.   .

$C_{22}H_{41}O_4N_3S$.   Calculated, C 59.55, H 9.33, found, C 59.83, H 9 10

From the curve it is obvious that the original sample was not homogeneous, and it is difficult to estimate exactly the percentage of the single major constituent.   The materials occurring both to the right and to the left of the central band were all active as antibiotics and probably represented substances as yet not characterized.   However, the partition coefficient calculated from the maximum point of the distribution probably
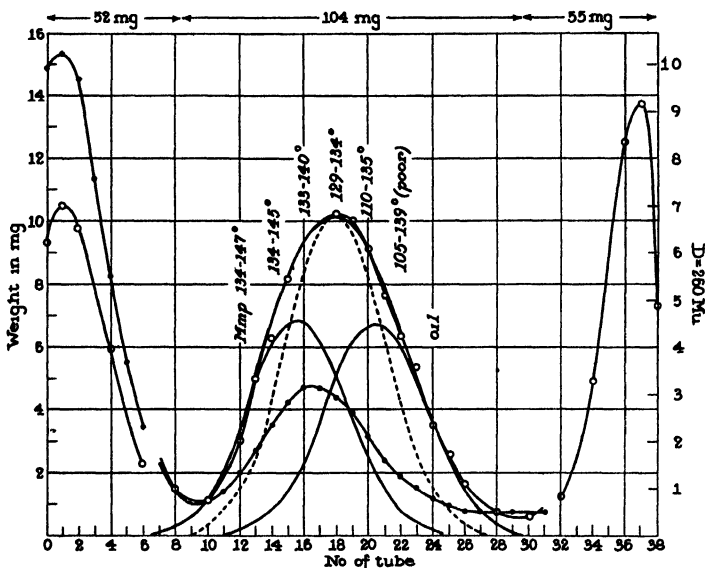
FIG. 7. Distribution pattern of mixture of four different penicillins; O = weight in mg. of triethylamine salt; ● = optical density at 260 mμ; solid line represents calculated curves; the dash line the calculated curve for one substance $K = 0.9$

is fairly reliable for use in characterizing the so called n-heptylpenicillin. It would appear to be 1.4 for the system and temperature used in the run.

A fair idea has now been obtained of the way these individual penicillins behave in the systems described, even though the samples used have been somewhat short of 100 per cent homogeneous.   Whether or not their presence together in the same sample in substantial quantities would affect the relative positions of the bands is a question which is all-important if the method is to be used in characterizing penicillins.   To answer this question a distribution was made in which 50 mg. of each of the four above crystalline sodium salts were added to Tube 0 at the start of the run.   The result of this distribution is shown in Fig. 7.

The procedure used in this distribution was somewhat different from that used in the previous ones. It will be described in full, since it involves a method of manipulation of the machine which has not as yet been described in previous publications. In this run the machine was filled and twenty-four transfers applied as in the previous runs. At this point the upper layer of Tube 0 was over the lower layer of Tube 24 and will be called Tube 0/24. The next tube in the clockwise direction was numbered 24/0. The lower layer of this tube contained the same buffer in which all the material was placed at the start of the distribution. The total material (both layers) in Tube 24/0 was withdrawn and numbered 0. It contained the material shown in Tube 0 of Fig. 7 and has had twenty-four transfers applied to it. Fresh equilibrated layers were then added to the empty tube of the machine and after equilibration an additional transfer was made. At this point the total material in Tube 0/0 of the machine was withdrawn and set aside. It contained the material which favored most the upper layer or had the highest partition coefficient. The previous tube withdrawn contained material with the lowest partition coefficient. The material now withdrawn had had twenty-five transfers applied to it and for purposes of plotting will be called Tube 38 in Fig. 7.

Fresh layers were then added to the empty tube of the machine, and another transfer applied. The contents of Tube 0/1 of the machine were withdrawn. This material corresponded to Tube 1 of Fig. 7 and had twenty-six transfers applied to it. The empty tube of the machine was refilled and another transfer applied. The contents of Tube 1/1 of the machine were then withdrawn and labeled 37 for Fig. 7. This material has had twenty-seven transfers applied to it. On the next withdrawal, Tube 1/2 gave Tube 2 of Fig. 7, while the next after that, Tube 2/2, of the machine, gave 36 of Fig. 7. This process could of course be continued indefinitely until all the solute in the machine had been withdrawn, but was here continued only until the material remaining in the machine had had a total of thirty-eight transfers applied to it. The procedure just described will in the future be called the procedure of "alternate withdrawal," and would appear to have considerable significance for the investigation of complicated mixtures.

At the end of the process described, the material remaining in the machine was withdrawn and numbered Tubes 7 to 31 consecutively, as shown in Fig. 7, Tube 6/7 corresponding to Tube 7 of the pattern. All of these tubes had had thirty-eight transfers applied, and a theoretical curve for matching could be fitted to this portion, just as in the routine runs used previously. ·Tubes 0 to 6 (material with the low partition coefficient, like the p-hydroxybenzylpenicillin) have had progressively twenty-four, twenty-six, twenty-eight to thirty-six transfers applied, and we do not at the present time have adequate mathematics developed for rapidly obtaining the theo-

retical weight which should be in each tube. It will be noted that a break occurs in the curve of Fig. 7 between Tubes 6 and 7 to show the change of procedure. Similarly the material in Tube 38 in reverse order to Tube 32 has had twenty-five, twenty-seven to thirty-seven transfers applied and contains almost all of the material of high partition coefficient, the n-heptylpenicillin.

Tube 37 readily gave the triethylammonium salt of n-heptylpenicillin, as shown by analysis, crystalline form, etc. Tube 1 gave the characteristic triethylammonium salt of p-hydroxybenzylpenicillin.

After withdrawal from the machine, addition of phosphoric acid, separation of the ether layers, and freezing in the dry ice jar, excess triethylamine was added to each of the tubes and the weight determined by evaporation to dryness. Evaporation to dryness of a triethylammonium salt in this manner appeared to give little if any loss of biological activity, and the various types could readily be crystallized from the residue.

The central broad band of Tubes 9 to 29 was not so clear cut at first glance. An attempt to fit a theoretical curve for a single substance resulted in the dotted curve shown, which is much too narrow. The next assumption from the symmetry of the broad experimental band is that it is composed of two substances in nearly equal amount. For such a case matching the experimental curve requires that a theoretical curve for each component must be found whose sum will fit the experimentally found curve. If the two are equal, it follows that the curves for the two components must intersect at a point midway to the maximum in Tube 18. The position of the maximum for each curve would then occur approximately at a point on the abscissa half way between this intersection and the point of the experimental curve at the same height (5.1 on the ordinate). This would approximate 15.5 for Component A and 20.5 for Component B. These data are sufficient for two theoretical curves to be calculated from the mathematics of the binomial expansion (12). Only slight adjustment of the two calculated curves places them so that their sum gives the theoretical curve to be matched with the experimental.

That this analysis of the composition of the band is not far from the correct one was demonstrated experimentally by the fact that the crystalline residue (the triethylammonium salt) from Tubes 11 to 14 agreed well in properties and in analysis for benzylpenicillin. The residue from Tubes 23 to 26 did not crystallize directly, in agreement with the previous experience with pure Δ^2-pentenylpenicillin. However, these residues did crystallize under the same conditions which induced pure Δ^2-pentenylpenicillin to crystallize and when seeded with that salt. Though the intermediate tubes crystallized directly, the melting points suggested mixed crystals even though the crystalline form appeared identical with pure benzylpenicillin.

Naturally a much better separation could have been achieved by application of more transfers, though for this it would be advisable to use more material at the *start*.

The mathematical analysis was further supported by the curve showing optical density at 260 mμ. The extinction coefficient of benzylpenicillin at 260 mμ is not a strong one, and the band plotted would appear to be slightly displaced toward the side of the Δ^2-pentenylpenicillin, due to overlapping by the absorption of the latter penicillin·which shows an extinction one-third that of benzylpenicillin at 260 mμ. This illustrates a serious limitation of the use of absorption spectrum as an analytical method.

That a reasonably accurate estimation of the relative amounts of benzyl- and Δ^2-pentenylpenicillin can be derived in such closely associated bands
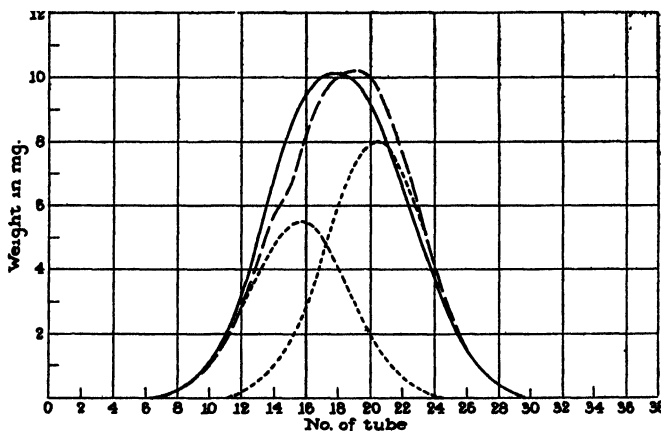
FIG. 8. Calculated distribution pattern for hypothetical mixture; dash line = 40:60 per cent mixture; solid line = 50:50 per cent mixture.

can be seen from Fig. 8. This chart shows the type of calculated curve to be expected from the sum of two curves of benzyl- and Δ^2-pentenylpenicillin when present in the hypothetical proportion of 40 and 60 per cent respectively.

Calculation of the partition coefficient from the maximum of 15.6 gives a figure of 0.7, while that of 20.4 gives the figure 1.16. The determined partition coefficients for benzyl- and Δ^2-pentenylpenicillin respectively are 0.75 and 1.18. The conclusion may therefore be drawn from these two curves and from the bands for p-hydroxybenzyl- and n-heptylpenicillin in Fig. 7 that the positions of the bands of the various penicillins are not greatly disturbed by the presence in the mixture of considerable quantities of other penicillins and that the constants established by study of the indi-

vidual ones alone are probably reliable in locating, for use in any future distribution, the positions of the bands of the four thus far studied.

A number of attempts were made to study the use of ethyl acetate as a solvent in this work. 1 M phosphate at a pH of 5.12 proved to be a suitable buffer for benzyl- and Δ^2-pentenylpenicillin. A summary of the results is given in Fig. 9 of a routine twenty-four transfer run made with benzylpenicillin in the same way as with ether. The main band agreed well with the calculated curve and one would ordinarily suspect a considerable amount of inhomogeneity in Tubes 0 to 6. The material in these tubes had very little
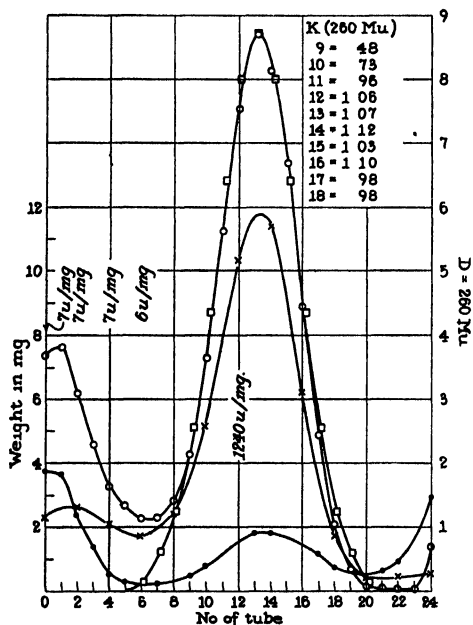
FIG. 9. Distribution pattern of benzylpenicillin. O = optical density at 260 mμ ● = optical density at 320 mμ; × = experimental weight curve; □ = calculated curve.

antibiotic activity. Upon further examination the suspected inhomogeneity was found to have a very low partition coefficient, much too low for it to migrate as far as Tube 5 or 6. A discrepancy is thus evident, and transformation of some sort during the run would be suspected. That this indeed was the case was shown by taking the material from Tubes 10 to 17, isolated as the crystalline triethylammonium salt, and distributing it again as in the original run. Again approximately 15 per cent of the same material appeared in Tubes 0 to 9 inclusive.

Attempts to distribute p-hydroxybenzyl- and Δ^2-pentenylpenicillin in

ethyl acetate both gave a similar result.   It is thus evident that the particular ethyl acetate at hand was not satisfactory for studying penicillin in a quantitative manner because of inactivation.   Whether or not a specially purified ethyl acetate would fail to cause inactivation can only be surmised at this point, but this is a problem we hope to take up at some time in the future.

From the above results it appeared possible to set up standards and constants which would be helpful in analyzing commercial penicillin prep-
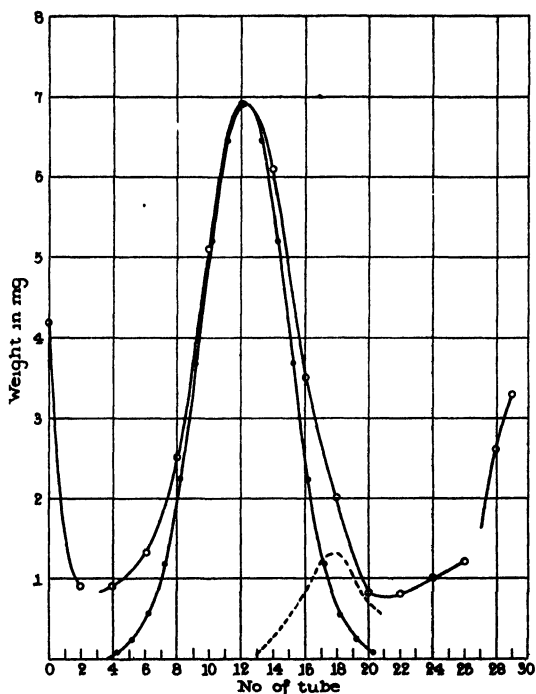
FIG 10 Distribution pattern of commercial penicillin; O = weight curve; ● = calculated curve for a single substance.

arations for clinical use as well as in definitely characterizing a particular penicillin.   Several routine distributions have therefore been made on commercial penicillin preparations available at the time the work was being done.   Most of these were amorphous.   None had activity against *Staphylococcus aureus* less than 1000 units per mg.   Four representative distributions of these runs in ether-2 M buffer at pH 4.8 are given in Figs. 10 to 13 inclusive.

The preparation shown in Fig. 10 had almost all its activity in the benzyl-
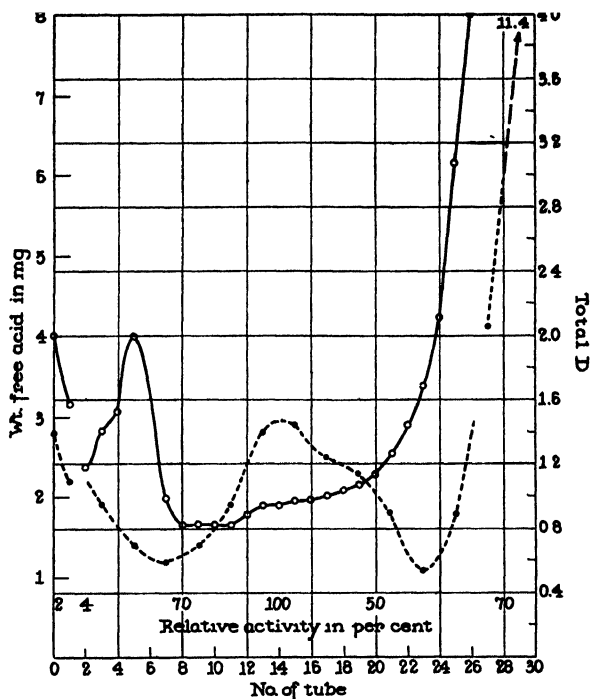
FIG. 11. Distribution pattern for a commercial penicillin; ○ = optical density at 260 mμ; ● = weight curve.
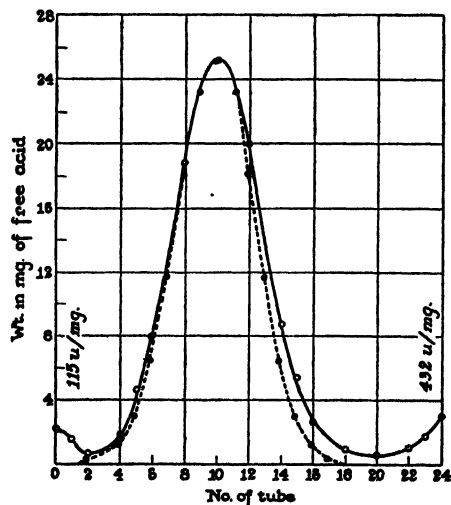
FIG. 12. Distribution pattern of a commercial penicillin; ○ = weight curve; ● = calculated curve.

penicillin band but contained active material in the Δ^2-pentenylpenicillin region, and probably contained approximately 5 to 10 per cent of the latter. Tube 11 of the main band gave readily the triethylammonium salt of benzylpenicillin, as shown by melting point, solubility, crystalline form, and analysis. The material in Tubes 0 to 2 as well as in Tubes 22 to 29 showed no antibiotic activity. As can be seen from the chart, the method of alternate withdrawal was used in this distribution.
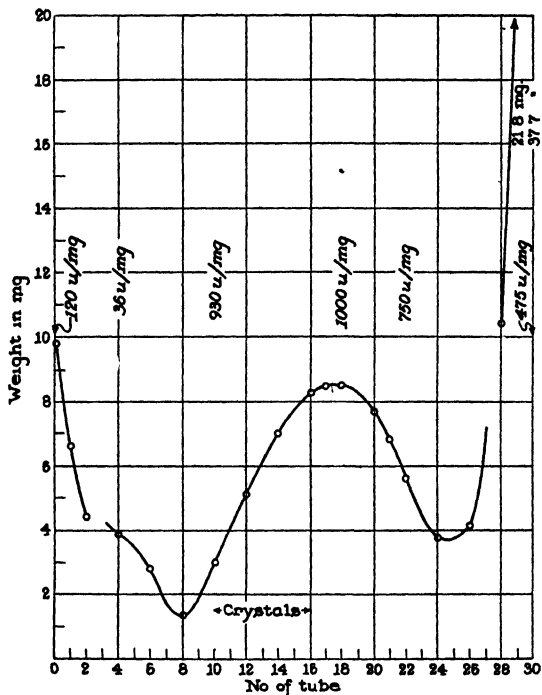
FIG. 13. Distribution of a commercial preparation of supposed high *n*-heptyl-penicillin content.

The distribution of Fig. 11 was run in exactly the same way, but showed a much more complicated picture. A small amount of crystalline triethyl-ammonium salt could be isolated from Tube 13, and presumably this is benzylpenicillin. The height of the curve at the position between Tubes 12 and 13 gives readily the maximum percentage of benzylpenicillin which could be present in the sample, since this point would correspond to the maximum of the benzylpenicillin band. A calculated curve for benzyl-penicillin should have a height at the maximum of 16.4 mg. The sample could not have, therefore, more than 11 per cent of benzylpenicillin in it, but probably contains somewhat less.

At least one other penicillin is present in the central overlapping band but, since this is too far displaced toward the right for Δ^2-pentenylpenicillin itself (this maximum should occur at 16), it is probable that another penicillin or a mixture of Δ^2-pentenylpenicillin and another penicillin is present. The high band to the right of Tube 24 is active antibiotically and contains material occurring in the n-heptylpenicillin region. The number of different penicillins present in this band has not been determined, but it would appear to represent more than 50 per cent of the total activity.

Fig. 12 would appear to be rather clear cut and to show practically no inactive material in the sample. It would appear to contain approximately 90 per cent of benzylpenicillin. Tube 9 readily gave the triethylammonium salt which agreed in melting point, solubility, crystalline form, antibiotic activity, and analysis with the triethylammonium salt of benzylpenicillin. Apparently there is present about 3 per cent of a n-heptyl type and 2 per cent of a partially active p-hydroxybenzyl type.

Fig. 13 again showed a much more complex picture and was rather similar to Fig. 11, except that it showed antibiotic activity in the p-hydroxybenzylpenicillin region. In this run triethylamine was added to each tube before evaporation. The only residues which crystallized directly were those in the benzylpenicillin band. The shape of the broad central band could scarcely be caused by benzylpenicillin and only one other penicillin. A minimum of three different antibiotics was most likely present in those tubes. A considerable amount of activity was also present in the large n-heptylpenicillin band occurring in Tubes 26 to 30 inclusive.

In order to learn something of the nature of the material in Tubes 26 to 30 another distribution was started in the same system in which more material was taken at the start. Only a few transfers were applied, but sufficient to separate most of the rapidly moving band in the end tube. The ether layer of this end tube which was 8 cc. in volume was then introduced as the upper layer in Tube 0 of a machine run which had been previously prepared by filling the apparatus with the system ether-1 M phosphate at pH 5.50. A routine twenty-four-stage distribution then gave the pattern shown in Fig. 14. Triethylamine was added before evaporation of each tube.

The material in Tubes 0 to 5 was probably the same as material occurring in the central tubes of Fig. 12 and was not examined further. The central band was much too wide to be formed from a single substance and contained at least two. Further, the approximately equal antibiotic activity of Tubes 10 and 18 indicated that a minimum of two antibiotics was present. Only Tubes 13 and 14 would crystallize under conditions favorable for the crystallization of the triethylammonium salt of n-heptylpenicillin and when seeded. This is the region of the maximum of the n-heptyl-

penicillin band. The material which resulted in Tubes 18 and 19 would appear to be an antibiotic not as yet described.

Considerable material appeared in Tubes 22 and 24 which was active antibiotically. Its nature and complexity would also appear to be unknown.

A certain amount of interest would appear to be attached to the information to be derived from Figs. 13 and 14, since they establish the
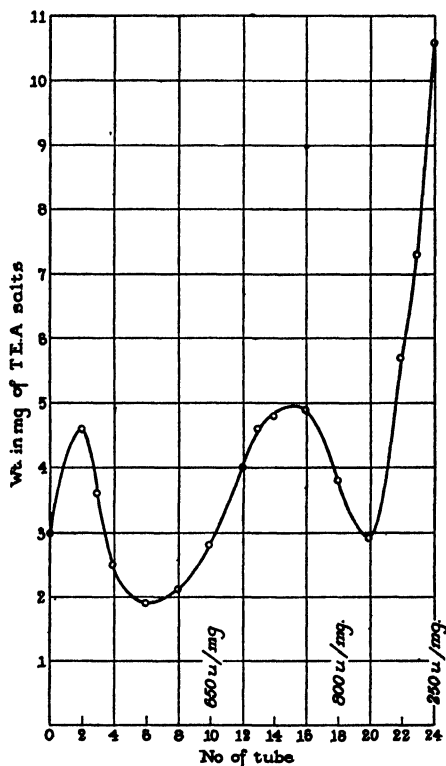
FIG. 14. Pattern of the redistribution of the n-heptylpenicillin band from Fig. 13. T. E. A. salts = triethylammonium salts.

fact that this particular penicillin preparation is quite heterogeneous with at least seven different penicillins being indicated in appreciable amounts. No single species could comprise more than 15 per cent of the total. This is a particularly interesting conclusion in view of the fact that this preparation was furnished us with the suggestion that it contained approximately 76 per cent of n-heptylpenicillin, as based on differential assay.

There would seem to be no doubt that the method of differential assay

has its value in estimating the relative amounts of penicillins when only two of the individual penicillins are known to be present and the identity of the two is known. This of course greatly limits its application. The method, however, when combined with the method of counter-current distribution, should make a very informative tool for the investigation of crude penicillins, since the latter method usually furnishes the preliminary simplification required for the first. This is a point we hope to follow further as opportunity presents itself.

It is only to be expected and is definitely indicated from Figs. 13 and 14 that the penicillins to be encountered in an amorphous preparation are by no means restricted to the four most common types. For this reason basic studies similar to the type reported in this paper will probably always be required, to deal with the problem intelligently.

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IDENTIFICATION OF SMALL AMOUNTS OF ORGANIC COMPOUNDS BY DISTRIBUTION STUDIES

V. CALCULATION OF THEORETICAL CURVES*

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The method of counter-current distribution for the purpose of fractionation, detection of inhomogeneity, or the characterization of an unknown compound has been described in several previous publications (1–3). Because of the nature of the process and the attainment of essential equilibrium at each step, the results are particularly adaptable to exact mathematical interpretation or analysis for any particular procedure chosen. Such mathematical interpretation is useful from the standpoint of regular practice as well as in understanding the underlying nature of such a process, and therefore is deserving of considerable attention. It is the purpose of the present treatment to deal with one phase of the interpretation, namely that of a machine distribution when nothing is withdrawn from the machine until the desired distribution is finished (Procedure 1 (1), or with the procedures given in the two later papers (2, 3)). These represent perhaps the simplest of all counter-current procedures and are therefore the most easy to interpret mathematically, since they require only the direct application of the binomial expansion (4) $(X+Y)^n$, where Y is considered the fraction being transferred in the upper layer or $K/(K+1)$ in terms of the partition coefficient and for equal volumes of the two phases. X is then the fraction remaining in the lower layer, $1-K/(K+1)$ or $1/(K+1)$. The expansion is therefore

$$\left[\frac{1}{K+1} + \frac{K}{K+1}\right]^n$$

Once such an actual distribution with a solute, homogeneous or otherwise, has been reached, the results can best be followed or interpreted by plotting a distribution curve. This curve is drawn by plotting the total amount of substance in each tube or cell of the machine *versus* the consecutive numbers on the tubes. Highly useful deductions can then be drawn from the general shape of the curve and the number of maxima obtained. For the proper interpretation of the curve, the theoretical distribution of a single

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pure substance having the same partition coefficient as the component in question is necessary and can be calculated directly from the binomial theorem. In this paper several simple and rapid short cuts are presented for the calculation of such theoretical distributions when the partition isotherm is a linear one.

The calculation of theoretical curves by a method of approximation has been referred to previously in the papers mentioned above. However, a direct application of the binomial expansion is necessary, when applied to relatively few transfers such as in eight transfer distributions, and for the higher distributions in which Tube 0 (or the highest tube) contains appreciable material. As the calculation depends only on the knowledge of the value of the partition coefficient of the pure solute in the system employed, methods of finding this value will be discussed later in the paper.

The problem presented is the calculation of the fraction of the pure ideal solute present in each of the tubes (both layers combined) after the distribution has been effected. From a step by step analysis of the binomial theorem, one is able to establish a table of general terms, Table I, giving the fraction of the original solute present in each tube at every stage of the analysis as a function of the partition coefficient, K.[1] The terms of Table I are those from the binomial expansion

$$\left[\frac{1}{K+1} + \frac{K}{K+1} \right]^n$$

Any single term, $T_{n,r}$, may be calculated directly from the mathematics of the binomial theorem, as given in Formula 1.

$$T_{n,r} = \frac{n!}{r!\,(n-r)!} \left(\frac{1}{K+1} \right)^n (K)^r \qquad (1)$$

$T_{n,r}$ is, therefore, the fraction of the original substance present in the Tube r in a distribution of n transfers or plates. K is the partition coefficient, or distribution constant, and is always defined as the concentration of solute in the upper phase divided by the concentration of solute in the lower phase. Thus formula (1) may be used to calculate a theoretical distribution for any given partition coefficient. For an eight transfer distribution, $n = 8$ and $r = 0, 1, 2, \cdots 8$, nine values must be calculated; for a twenty-four transfer distribution, $n = 24$, $r = 0, 1, 2, \cdots 24$, twenty-five values must be calculated.

[1] All formulae derived in this paper will apply only to distributions in which the upper phase migrates, as in a machine distribution. They will apply to distributions in which the lower phase migrates if $1/K$ is substituted for K. Also the volumes of the two phases are assumed to be equal. When they are not equal, the calculation may be done by substituting the product $K \times r$ wherever K is used. r in this case is the ratio of the upper and lower volumes.

TABLE I

Terms of Binomial Expansion*

$n \downarrow$ \ r	0	1	2	3	4	5	6	7	8
0	1								
1	$\dfrac{1}{K+1}$	$\dfrac{K}{K+1}$							
2	$\dfrac{1}{(K+1)^2}$	$\dfrac{2K}{(K+1)^2}$	$\dfrac{K^2}{(K+1)^2}$						
3	$\dfrac{1}{(K+1)^3}$	$\dfrac{3K}{(K+1)^3}$	$\dfrac{3K^2}{(K+1)^3}$	$\dfrac{K^3}{(K+1)^3}$					
4	$\dfrac{1}{(K+1)^4}$	$\dfrac{4K}{(K+1)^4}$	$\dfrac{6K^2}{(K+1)^4}$	$\dfrac{4K^3}{(K+1)^4}$	$\dfrac{K^4}{(K+1)^4}$				
5	$\dfrac{1}{(K+1)^5}$	$\dfrac{5K}{(K+1)^5}$	$\dfrac{10K^2}{(K+1)^5}$	$\dfrac{10K^3}{(K+1)^5}$	$\dfrac{5K^4}{(K+1)^5}$	$\dfrac{K^5}{(K+1)^5}$			
6	$\dfrac{1}{(K+1)^6}$	$\dfrac{6K}{(K+1)^6}$	$\dfrac{15K^2}{(K+1)^6}$	$\dfrac{20K^3}{(K+1)^6}$	$\dfrac{15K^4}{(K+1)^6}$	$\dfrac{6K^5}{(K+1)^6}$	$\dfrac{K^6}{(K+1)^6}$		
7	$\dfrac{1}{(K+1)^7}$	$\dfrac{7K}{(K+1)^7}$	$\dfrac{21K^2}{(K+1)^7}$	$\dfrac{35K^3}{(K+1)^7}$	$\dfrac{35K^4}{(K+1)^7}$	$\dfrac{21K^5}{(K+1)^7}$	$\dfrac{7K^6}{(K+1)^7}$	$\dfrac{K^7}{(K+1)^7}$	
8	$\dfrac{1}{(K+1)^8}$	$\dfrac{8K}{(K+1)^8}$	$\dfrac{28K^2}{(K+1)^8}$	$\dfrac{56K^3}{(K+1)^8}$	$\dfrac{70K^4}{(K+1)^8}$	$\dfrac{56K^5}{(K+1)^8}$	$\dfrac{28K^6}{(K+1)^8}$	$\dfrac{8K^7}{(K+1)^8}$	$\dfrac{K^8}{(K+1)^8}$

*Table I is representative of distributions in which the upper phase migrates.

Rather than calculate each value independently from equation (1), more rapid methods are possible which in principle determine only one term directly and calculate all other terms from this one. For a specific expansion, i.e. for a given value of n, adjacent terms of the expansion are very simply related to one another. This is evident upon examination of Table I, e.g.

$$(n = 8)$$

$$T_1 = 8KT_0$$

$$T_2 = \frac{7}{2} KT_1 , \text{ etc.} \tag{2}$$

In general terms, the rth term is related to the $(r-1)$th and the $(r+1)$th term by formulae (3) and (4) respectively.

$$T_r = FKT_{r-1} \tag{3}$$

$$T_r = F' \left(\frac{1}{K}\right) T_{r+1} \tag{4}$$

where $F = (n+1-r)/r$ and $F' = (r+1)/(n-r)$.

For eight and twenty-four transfer distributions the factors F and F' are given in Table II. A direct application of the use of these factors is discussed later in this paper. For eight transfer distributions in which the lower phase migrates, a further convenience results if a series of nine graphs are plotted from equation (1), ($1/K$ substituted for K) with T_r as the ordinate and K as the common abscissa, for values of $r = 0, 1, 2, \cdots 8$. From these graphs one is able to read all nine values, one from each curve, for any value of K, and hence obtain the theoretical concentrations in a very few minutes. These curves are given in Fig. 1 for values of K from 0.40 to 2.50.

As T_r is the fraction of the original solute in Tube r, it is numerically equal to the amount of solute in gm. only provided the experiment was begun with 1 gm. In general, experiments are not begun with a unit weight, so that to determine the actual amount present in Tube r the value T_r must be multiplied by the total weight of solute used.

In applying these data to the interpretation of an actual distribution, i.e. the fitting of a curve, it is well to emphasize the basic point, namely that for a pure solute and a given number of transfers the relative amount of substance in each tube is fixed (assuming a linear isotherm). Thus in an inhomogeneity analysis, if the values of T_r are multiplied by the weight of the inhomogeneous sample, the experimental concentration values for the tubes at the extremities of the distribution will exceed the theoretical ones by nearly the actual amount of the impurity present, while the theoretical

<div align="center">

TABLE II

Factors for Eight and Twenty-Four Transfer Distributions

</div>

r	8 transfer distribution		24 transfer distribution	
	F	F'	F	F'
0		1/8	·	1/24
1	8	2/7	24	2/23
2	7/2	3/6	23/2	3/22
3	6/3	4/5	22/3	4/21
4	5/4	5/4	21/4	5/20
5	4/5	6/3	20/5	6/19
6	3/6	7/2	19/6	7/18
7	2/7	8	18/7	8/17
8	1/8		17/8	9/16
9			16/9	10/15
20			5/20	21/4
21			4/21	22/3
22			3/22	23/2
23			2/23	24
24			1/24	
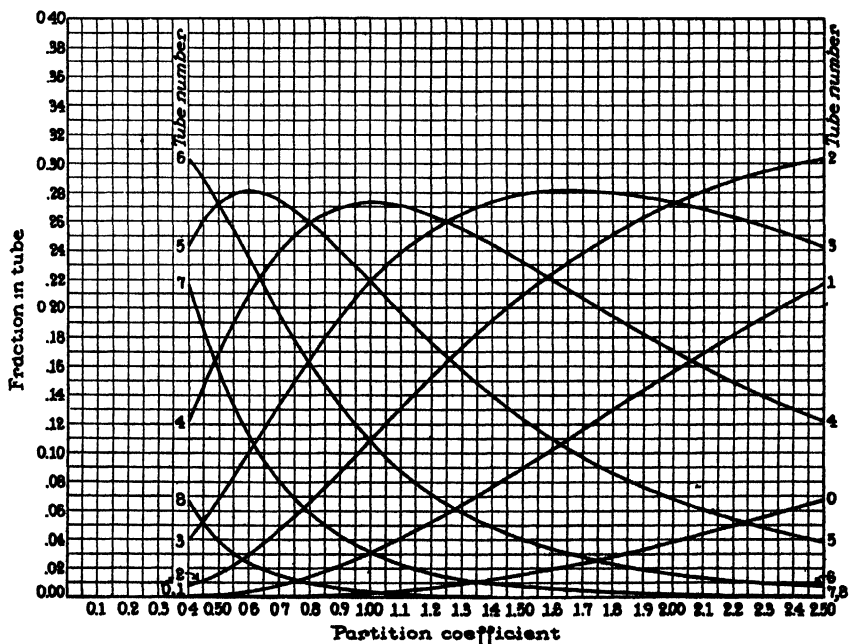
FIG. 1. Graph of eight transfer distribution for different partition coefficients

values for the central tubes will exceed the experimental ones by a proportional amount.

The pure solute from the inhomogeneous sample will usually be concentrated in the tubes comprising the peak of the distribution, and it is thus valid to attempt to fit the maximum of the theoretical curve to that of the experimental one. Impurities are then quantitatively indicated by the deviations at the extremities of the two curves, the experimental values always exceeding the theoretical ones by the amount of impurity present. This fit is accomplished by multiplying all T_r values by the factor $T'_{max.}/T_{max}$, where $T'_{max.}$ is the experimental value for the tube of maximum concentration and $T_{max.}$ is the theoretical value for this same tube on the basis of unity, *i.e.* as calculated from formula (1), or as read from Fig. 1. In the event the means of analysis does not result in the direct determination of the actual weight of substance in each of the tubes (*e.g.* spectroscopic analysis), the figures proportional to weight may be used.

In practice, however, especially with distributions of more than eight transfers matching the theoretical curve to the experimental one is best accomplished directly by the procedure now to be outlined. After determining the experimental distribution, a value is chosen near the peak of the distribution curve, preferably the maximum one, and, assuming this to lie on the theoretical curve, the other values composing the theoretical distribution are calculated by use of the factors F and F'. The use of the point near the maximum of the distribution curve involves the previously stated assumption that the solution represented by this point is that of a pure compound.

If a distribution is attempted with a mixture of two substances whose partition coefficients are close together, for example a mixture of isomers, a clear separation may not result, and instead of obtaining a curve of two distinct peaks a rather broad, single peak may occur. In attempting to fit a theoretical curve to such a distribution, one must assume the existence of at least two substances and find two curves which, when added together, will give the experimental one. The application of this method to sterioisomers, diasterioisomers, and other very closely related compounds is being undertaken and appears to offer promise. Methods for a more complete separation of compounds with nearly identical partition coefficients may also be found in variations of the standard distribution procedures. Such possible variations are being further investigated. Two typical curves are given in Figs. 2 and 3. Fig. 2 is that of an eight transfer distribution of a mixture of two compounds consisting of 90 per cent of Compound A ($K = 1$) and 10 per cent of Compound B ($K = 10$). Fig. 3 is a twenty-four transfer distribution of the same mixture, showing the increased separation rendered by the use of a greater number of transfers. In all cases in which

an applied theoretical curve will not coincide with the experimental one, it is indicated by a dotted line.

By use of the above methods, the theoretical distribution of a pure substance can be determined exactly for any given partition coefficient.
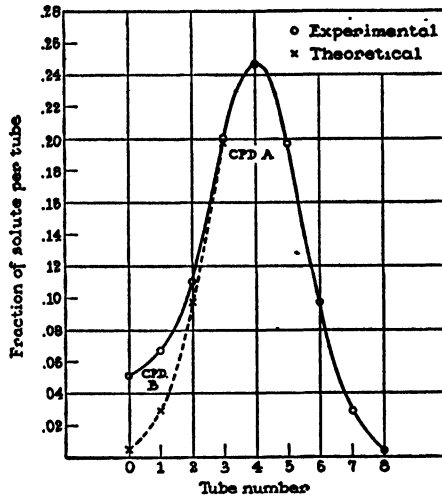
FIG. 2. Pattern of eight transfer distribution
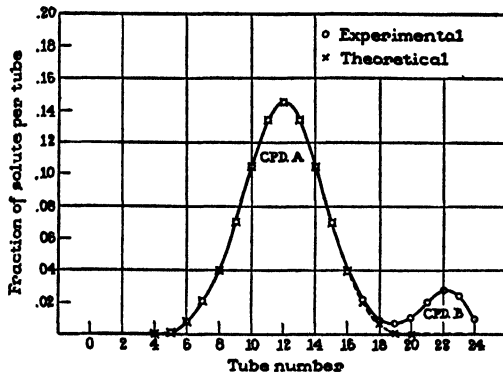
FIG. 3. Pattern of twenty-four transfer distribution

Therefore in contrasting theoretical concentrations with those obtained experimentally in an inhomogeneity analysis, the partition coefficient of the pure substance must be determined. The ways in which this may be achieved are several.

Direct Measurement—The partition coefficient as measured before the distribution is that of the inhomogeneous solute, and is consequently not

usable in the theoretical determination. The material in the tubes near the peak of the experimental distribution, however, should be the most nearly homogeneous, except in the case of an inhomogeneity with an identical or nearly identical partition coefficient. These solutions may thus be used to determine experimentally the partition coefficient of the pure solute, unless otherwise indicated by the shape of the curve. Direct measurement is always used in this laboratory to serve as a check on experimental procedure or calculations, as well as on possible deviations from a linear partition isotherm; but for use in the calculation of a theoretical curve to be fitted K may be derived as given below, without the separate experimental determination.

Indirect Calculation—When the tubes near the peak of the curve contain homogeneous material, this part of the experimental curve is representative of the true partition coefficient of the pure solute. Further, the form and position of this part of the curve are quite sensitive to the value of the partition coefficient, and herein lies the basis for several methods of determining this coefficient. Two are mentioned here.

A previously reported application (1) of this principle is in the use of the formula

$$N = n\left(\frac{K}{K+1}\right) \tag{5}$$

which defines the relationship between the position N of the peak of the curve and the partition coefficient, K, where n is the number of transfers in the distribution. This formula assumes a continuous function and is exact only if K is equal to unity or if there are an infinite number of transfers in the distribution. For distributions involving a number of transfers greater than twenty, when the partition coefficient is near unity, the above formula is satisfactory in ordinary practice. Its disadvantages are 2-fold; namely, the abscissa of the peak of the curve, N, can only be estimated rather than determined accurately, and secondly, it cannot be applied to eight transfer distributions.

A second method of determining the partition coefficient from the region near the peak or from any other part of the experimental distribution curve utilizes the condition that the ratio of the concentrations in any two tubes is a specific function of the partition coefficient. The concentration in any one tube is a function of both the initial concentration and the partition coefficient. The ratio of the concentrations in any two tubes, however, is a function of the partition coefficient only, and is independent of the initial concentration. For example, in an eight transfer distribution, $T_4/T_5 = 5/4(K)$. Hence

$$K = [5/4(T_4/T_5)] \tag{6}$$
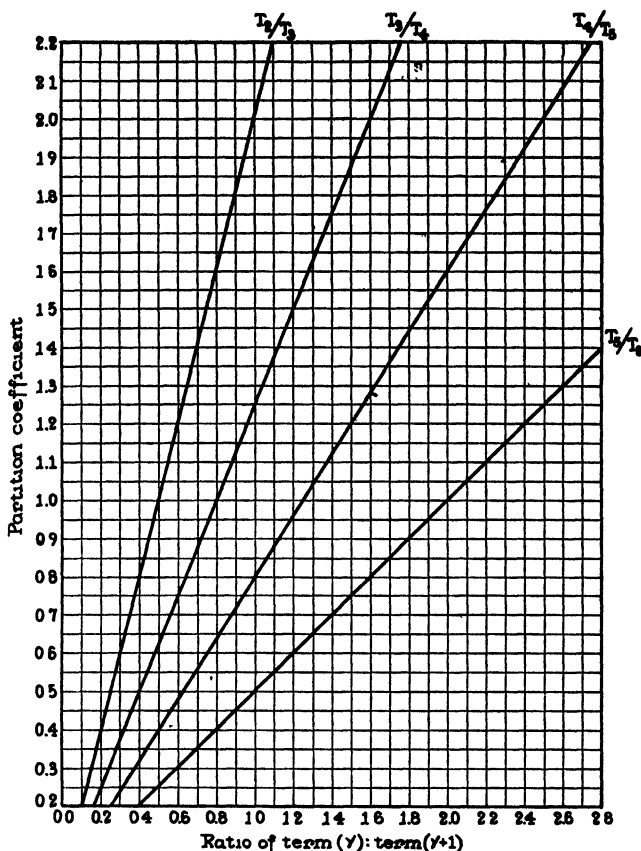
FIG 4   Graph for determining partition coefficient

In general, from equations (3) and (4),

$$K = \frac{1}{F}\frac{T_r}{T_{r-1}} \tag{7}$$

$$K = F'\frac{T_{r+1}}{T_r} \tag{8}$$

If the various ratios, T_r/T_{r+1}, are plotted against K, straight lines will be obtained (Fig. 4) and from such graphs the partition coefficient can be read quickly and accurately.   Thus through the use of the graphs of Figs. 1 and 4 an eight transfer theoretical distribution may be quickly calculated and applied.

The methods given in this paper for the calculation of theoretical curves

$$\frac{dc}{dx} = \frac{C}{\sqrt{a\pi \left(\frac{K}{K+1}\right)^n}} \, e^{-x^2/a \left(\frac{K}{K+1}\right)n} \tag{9}$$

will supersede in many instances the formula earlier derived for the calculation of theoretical curves. Equation (1) is exact, while equation (9) is an approximation. Nevertheless, equation (9) is of interest because it permits easy calculation of curves for numbers of transfers higher than twenty-five. Further, it is apparent that very close approximations may be made with this type of mathematical calculation. In Fig. 5 curves for $K = 0.707$
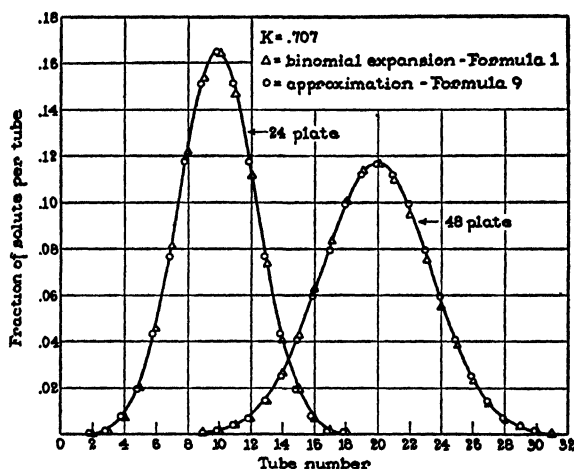
FIG. 5. Agreement of calculation with the exact calculation and the approximation

are given for twenty-four and forty-eight plate distributions calculated exactly by formula (1) and by the approximation of formula (9). The constant a, which was derived empirically, approaches 2 for either large or small coefficients, since it is equal to $2(1/K+1)$. Here the reciprocal of K, $1/K$, must be used when K is greater than 1. Thus equation (9) becomes equation (10)

$$y = \frac{1}{\sqrt{2\pi \frac{K}{(K+1)^2} n}} \, e^{2 \frac{-x^2}{\frac{K}{(K+1)^2} n}} \tag{10}$$

where y is the ordinate or the fraction of the substance in the tube in question, K is the partition coefficient, n is the number of transfers, and x is the number of tubes between the one in question and the maxima.

˙ Thus when x is 0, y is the maximum. Equation (9) is in accord with previous equations (5) for this type of calculation and coincides with the curve of error. ⁿ

Our theoretical considerations have thus far been restricted to the simplest possible type of manipulation of the machine. In this no attempt is made to prevent the band from spreading or otherwise to modify it, such as periodic withdrawal of certain fractions, evaporation to dryness, and return to the preceding tube would achieve. This latter procedure offers the possibility of one way of introducing a principle with the same effect in our machine that the principle of reflux has, as used in fractional distillation. Also the periodic withdrawal of certain fractions offers itself certain interesting possibilities. It is our intention to discuss these approaches in a forthcoming paper when more experimental data are at hand.

SUMMARY

Methods for rapid and accurate calculation of theoretical curves for use in the "counter-current distribution" method have been presented.

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FLUOROPHOTOMETRIC ESTIMATION OF STILBAMIDINE IN URINE AND BLOOD

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Stilbamidine (4,4'-diamidinostilbene) has been introduced by Yorke and his colleagues (1) as a powerful chemotherapeutic agent for the treatment of trypanosomiasis, kala-azar, and babesiasis. It has also been experimentally used in the therapy of purulent arthritis (2), simian malaria (3), and most recently in multiple myeloma (4). A method for the estimation of this drug in biological fluids is of importance and is the basic requirement of any study with stilbamidine. Direct application of physical methods, *i.e.* fluorescence or ultraviolet absorption, while highly desirable because of their sensitivity and specificity, results in inaccurate readings because of the presence in biological fluids of interfering substances. In the method to be presented stilbamidine is first separated quantitatively by an ion exchange column of Decalso (suggestion of Dr. Oliver Lowry), and then eluted and measured with a fluorophotometer. The method also has the advantage that a preliminary precipitation of plasma proteins is not necessary.

Principle—Stilbamidine exhibits a brilliant blue fluorescence when exposed to ultraviolet light and this property can be used for its estimation. The fluorescence is measured by a photometer after separation from other fluorescent materials. The fluid to be examined is placed on a column of Decalso for adsorption. The column is washed free of impurities with large volumes of hot water. Elution is carried out with a 0.2 N HCl-50 per cent ethanol mixture, which is more efficient than the usual HCl-KCl mixture. The small blank value for the urine is then reduced by strong acidification of the eluate. After a specific time interval the eluate is measured in a fluorophotometer.

Reagents

1. Decalso, 50 to 80 mesh, washed before use with 3 per cent acetic acid followed by distilled water and drying.

2. Silica sand, fine granular.

3. Eluting mixture, equal parts of 0.4 N HCl and 95 per cent ethanol.

4. Hydrochloric acid, c.p., concentrated.

Method of Analysis

Stilbamidine is photolabile in aqueous solution, necessitating the performance of all stages of the method in dim artificial light or in the dark. Adsorption columns were made from ordinary glass tubing 9 mm. in diameter, with a 0.5 mm. hole at the bottom. They should be at least 10 inches long and preferably with a funnel top. A few grains of silica sand are added to the column which is wetted with a drop of water applied to the bottom. Decalso is added until a column 6 inches in length is formed. Gentle tapping of the tube assures uniform distribution of the Decalso.

Urine samples are collected in dark bottles. Measure 5 cc. of urine (containing 1 to 30 γ of stilbamidine per cc.) into the column and allow adsorption by gravity. It is advisable to check adsorption by a momentary exposure to a weak source of ultraviolet light.

Wash the column with 60 cc. of hot water, using gentle suction. Discard the water. Elute with three portions of 4 cc. of eluting mixture and collect in a 25 cc. volumetric flask. This step is done without suction.

Add 1 cc. of concentrated HCl to the solution in the flask, dilute to the mark with distilled water, mix, and place in the dark for 15 minutes before measuring the fluorescence.

Standard Solutions—The stock standard is a 30 mg. per cent aqueous solution of stilbamidine isethionate. This is stable in the dark at room temperature, losing only 2 per cent per week. Of this solution 0.1, 0.3, and 0.5 cc. are carefully measured into 25 cc. volumetric flasks. 12 cc. of elution mixture are added to each flask and mixed. Then 1 cc. of concentrated HCl is added and the solution made up to the mark with distilled water. After mixing, exactly 15 minutes should elapse before the fluorescence is measured. Standard solutions in water are not comparable, as they have only two-thirds of the fluorescence of those containing alcohol.

Reagent Blank—12 cc. of elution mixture and 1 cc. of concentrated hydrochloric acid are made up to 25 cc. with distilled water.

Fluorometric Measurement—An instrument containing a balance photocell and bridge circuit is required for stable, reproducible readings and linear response (Lumetron[1]). The fluorescence is measured with the usual primary and secondary vitamin B_1 filters. and a wire screen which cuts down the intensity of the measuring beam about 70 per cent. After the Lumetron has warmed up. the secondary standard (1 γ per cc. of quinine sulfate in 0.1 N sulfuric acid) is set at 50 and the reagent blank at 0 with the zero suppressor control. If the 15 cc. sample holder is used, 12 cc. aliquots are pipetted into the sample holder.

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[1] Photovolt Corporation, New York.

EXPERIMENTAL

The fluorescence of stilbamidine deteriorates rapidly during measurement, probably owing to saturation of the ethylene linkage (5). The deterioration experienced under our conditions of measurement is given in Fig. 1. It is seen that the greatest rate of change of fluorescence is in the 1st minute. If one exposes the solution to be examined for exactly 1 minute before taking a reading, reproducible measurements can be made with a variation of 2 per cent or less. The disadvantage of reading the
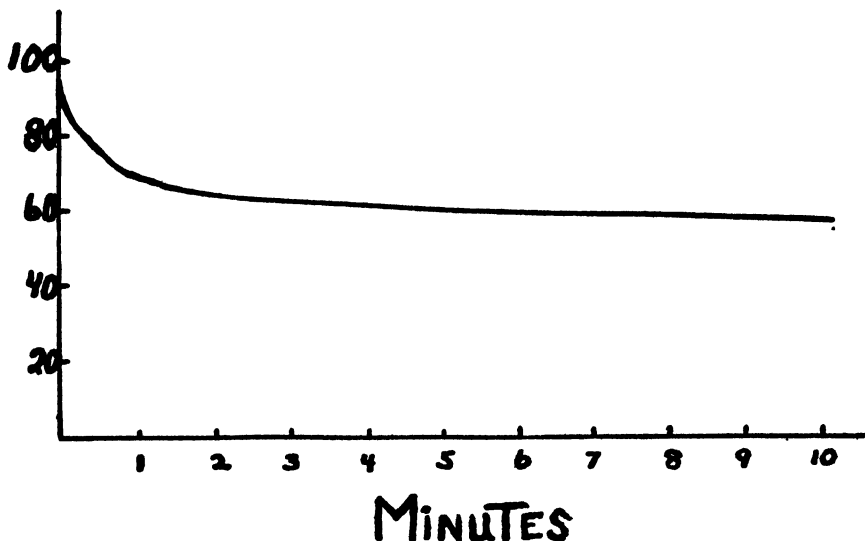
FIG. 1. Galvanometer readings of stilbamidine fluorescence

fluorescence after a longer period of time is that the fluorescence of the blank does not change, so that proportionately it becomes greater.

A graph of the values for the fluorescence of the standard solutions forms a straight line for the low concentrations measured (Fig. 2). It can be noted that a reading of 60 is obtained when 0.09 mg. of stilbamidine isethionate was present in the original 5 cc. aliquot of urine, the blank on the urine without stilbamidine being, on the average, about 2. By this method the drug content of the urine or other biological fluids can be measured accurately down to 1 γ per cc. of fluid tested.

Recoveries averaging 87 per cent were obtained in experiments in which stilbamidine was added to 5 cc. samples of urine (Table I). The average deviation of a single determination was less than 2 per cent. This is a satisfactory result for a method employing Decalso, the loss being due to an inseparable, permanently adsorbed fraction.

TABLE I

Recovery of Stilbamidine Isethionate

		Stilbamidine added	Per cent recovered*
		mg.	
Urine	1	0.015	96
"	2	0.03	89
"	3	0.06	88
"	4	0.06	87
"	5	0.09	87
"	6	0.09	87
"	7	0 09	84
"	8	0 09	86
"	9	0.09	83
"	10	0.09	87
"	11	0.09	85
"	12	0.12	85
"	13	0.12	87
Plasma	1	0 018	84
"	2	0.018	85
"	3	0.036	88
"	4	0 06	89

* After subtraction of the blank.
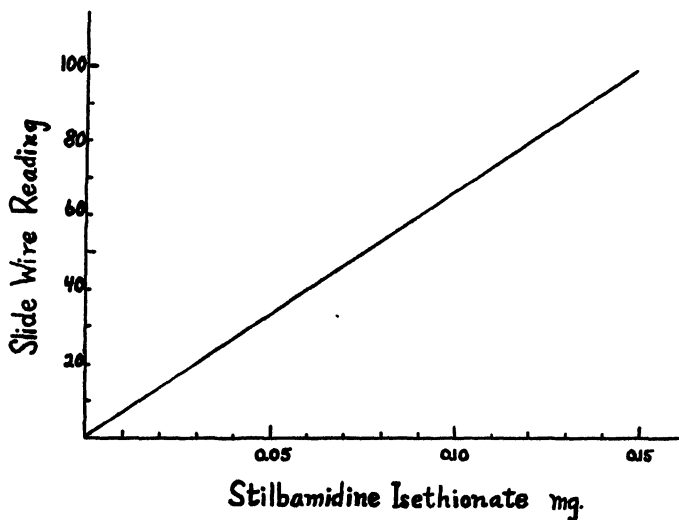
FIG. 2. Fluorescence curve of stilbamidine

Proteins need not be removed before placing the plasma on the Decalso column. A procedure similar to that for urine is applied to 2 cc. samples of plasma. The quinine standard is set at 100 and weaker standard solu-

tions are necessary. Blank values of plasma without stilbamidine are negligible. Recovery of stilbamidine added to plasma was the same as with urine (Table I). Culture media containing stilbamidine can be examined in the same way.

Comment

The pharmacological and clinical studies of stilbamidine and other aromatic amidines have been handicapped by a lack of suitable methods for the estimation of the amounts of these drugs in urine and blood. By making use of the two readily apparent properties of stilbamidine, adsorption and fluorescence, a simple and accurate method for the determination of stilbamidine in biological fluids was evolved. Recently a method has appeared for the fluorometric determination of aromatic amidines based upon the reaction of the latter with glyoxal and benzaldehyde in alkaline aqueous solution with formation of fluorescent glyoxalidone derivatives (6). Although this is a definite improvement over previous methods employing glyoxal, it requires the separation of amidine from plasma proteins with either dialyzed iron or butyl alcohol, and for urine samples there are frequently (6) "extraneous compounds which inhibit the reaction of the aromatic amidines with glyoxal and benzaldehyde." Furthermore, the range to be measured (2 to 10 γ of stilbamidine) is more restricted than with the Decalso method.

SUMMARY

A simple and accurate method of estimation of stilbamidine in urine and blood is given. The stilbamidine is separated from interfering substances by adsorption on a column of Decalso, which is then washed with hot water. Preliminary protein precipitation is not necessary. Elution is carried out with a hydrochloric acid-ethanol mixture, which is further acidified to reduce blank fluorescence. The characteristic blue fluorescence of the stilbamidine is then measured in a fluorophotometer.

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A CHEMICAL METHOD FOR THE DETERMINATION OF PTEROYLGLUTAMIC ACID AND RELATED COMPOUNDS

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WITH THE TECHNICAL ASSISTANCE OF ANNA DE GRUNIGEN

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Pteroylglutamic acid, N-[4-{[(2-amino-4-hydroxy-6-pteridyl)methyl]-amino}benzoyl]glutamic acid, on either chemical or catalytic reduction at
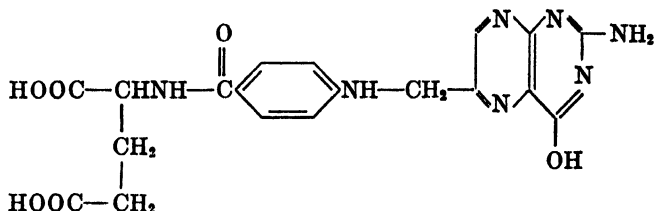
an acid pH is cleaved to yield a pteridine and p-aminobenzoylglutamic acid.[1] The reaction seems to be general for this group of compounds and is suggested as a basis for a chemical method for the determination of pteroylglutamic acid, its derivatives, and analogues.

The method consists of reducing the compound with zinc dust in 0.5 N hydrochloric acid. The aromatic amine is measured by the method of Bratton and Marshall (1) before and after reduction. The difference between the two values multiplied by the appropriate factor is a measure of the pteroyl derivative present.

Method

Apparatus—A photoelectric colorimeter is necessary to measure accurately the color formed in the Bratton and Marshall procedure. An Evelyn photoelectric colorimeter and a Beckman spectrophotometer have been used.

Reagents—5.0 N hydrochloric acid.

Reagent grade of zinc dust.

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[1] Hutchings, B. L., Stokstad, E. L. R., Mowat, J. H., Boothe, J. H., Waller, C. W., Angier, R. B., Semb, J., and SubbaRow, Y., unpublished data.

0.5 per cent solution of gelatin containing 0.1 per cent benzoic acid as a preservative.

0.1 per cent solution of sodium nitrite.

0.5 per cent solution of ammonium sulfamate.

0.1 per cent solution of N-(1-naphthyl)-ethylenediamine dihydrochloride. This reagent is light-sensitive and should be kept in a brown bottle.

0.1 per cent solution of p-aminobenzoic acid in 50 per cent ethanol.

Procedure

A general outline of the method, with pteroylglutamic acid as an example, follows. A solution containing from 0.5 to 3.0 mg. of pteroylglutamic acid is placed in a 100 ml. volumetric flask. To this flask are added approximately 80 ml. of water, 10 ml. of 5.0 N hydrochloric acid, 1.0 ml. of a 0.5 per cent solution of gelatin, and sufficient water to make the total volume 100 ml. A sample of this unreduced solution, usually 2 ml., is removed and used for determining the free amine. If the original compound or crude product contains less than 2 per cent of free amine, an aliquot of the original solution must be used in order to obtain a readable color.

The remainder of the solution is transferred to a 250 ml. Erlenmeyer flask and reduced by the addition of from 0.5 to 1.0 gm. of zinc dust. The amount of zinc dust is not critical and can be conveniently estimated once a weighed amount has been used. After 10 minutes reduction, during which time the flask is shaken intermittently, the zinc dust is filtered off and the amine is determined on 2 ml. of the filtrate by the following procedure: The sample is diluted to 6.6 ml. with water. To this solution are added, with thorough mixing, 0.4 ml. of 5.0 N hydrochloric acid and 1.0 ml. of sodium nitrite solution. After 3 minutes, 1.0 ml. of ammonium sulfamate solution is added and the decomposition of the excess nitrous acid is allowed to proceed for 2 minutes. At the end of this period, 1 ml. of N-(1-naphthyl)-ethylenediamine solution is added. The color reaches a maximum in 5 minutes and is stable for several hours. The color may be measured at 550 mμ in a Beckman or Coleman spectrophotometer or in a photoelectric colorimeter with a 550 mμ filter. The use of specially cleaned cuvettes decreases the tendency of bubbles of nitrogen gas to adhere to the sides of the cuvettes.

Since p-aminobenzoic acid, p-aminobenzoylglutamic acid, and p-aminobenzoylglycine give the same molal color, it is reasonable to suppose that other peptides of p-aminobenzoic acid would give similar results. On the basis of this assumption p-aminobenzoic acid has been used as a standard in the Bratton and Marshall method. A response curve is constructed with from 5 to 20 γ of p-aminobenzoic acid. This is necessary since the color intensity does not exactly follow Beer's law.

Calculations—The difference between the amine obtained after reduction

and the amine obtained on the unreduced solution yields the combined amine present. This value multiplied by the factor, (molecular weight of pteroylglutamic acid)/(molecular weight of p-aminobenzoic acid), gives the micrograms of pteroylglutamic acid in the diluted sample.

<div align="center">EXPERIMENTAL</div>

Extent of Reduction—The magnesium salt of pteroylglutamic acid was prepared by heating the free acid with magnesium oxide in the minimum amount of water. The solution was filtered to free it of excess magnesium oxide. On cooling, magnesium pteroylglutamate crystallized as needles. After three recrystallizations the magnesium salt was collected, air-dried, and then dried in a high vacuum at 110° for 4 hours.

$C_{19}H_{16}O_6N_7Mg_{1.5} \cdot H_2O$
 Calculated. C 46.29, H 3.66, N 19.90, Mg 7.41
 Found. " 46.51, 46.44, " 4.20, 3.70, " 19.92, 19.78, " 7.38, 7.29

When the compound was reduced under the conditions described above, it was found to contain 0.09 per cent free amine as p-aminobenzoic acid and 28 25 per cent total amine. The difference between these two values multiplied by the factor, $(C_{19}H_{16}O_6N_7Mg_{1.5} \cdot H_2O)$/(molecular weight of p-aminobenzoic acid), gives a figure of 101.2 per cent. As the accuracy of the Bratton and Marshall determination is ±2 per cent, the figures indicate that the reductive cleavage is essentially complete.

An independent method of assessing the purity of the compound was based on a comparison of the extinction coefficients of the magnesium pteroylglutamate with the value for pteroylglutamic acid. The $E_{1\,cm.}^{1\%}$ value at 365 mμ for magnesium pteroylglutamate was 184. When this is corrected for the magnesium and water content, the $E_{1\,cm}^{1\%}$ value is 205.5. The value for a highly purified preparation of the free acid is 206. This is further evidence for the essentially complete reduction of pteroylglutamic acid into its pteridine and aromatic amine components.

Time of Reduction—Samples were reduced for the lengths of time noted in Table I. Maximum amine liberation occurred in less than 10 minutes. Further reduction, especially with certain samples of zinc dust, led to somewhat lower values. This apparently arises from partial destruction of the aromatic amine that is formed on reductive cleavage (see below).

Stability of p-Aminobenzoic Acid and p-Aminobenzoylglutamic Acid to Reduction—p-Aminobenzoic acid and p-aminobenzoylglutamic acid were reduced under conditions similar to those used for the pteroyl derivatives. Definite destruction of the aromatic amines occurred (Table II). The inclusion of gelatin in the reducing solution protects the amines from inactivation. The exact nature of this protective action is unknown.

Stability of Diazo Compound to Light[2]—A series of experiments was carried out in which p-aminobenzoylglutamic acid was diazotized in red flasks which were non-actinic, in ordinary glassware in laboratory light (slight

TABLE I

Effect of Time and Acidity on Reduction of Air-Dried Pteroylglutamic Acid by Zinc

Time	Acid concentration	p-Aminobenzoic acid	
		1st determination	2nd determination
min.		*per cent*	*per cent*
1	0.5 N HCl	28.9	28.9
2	0.5 " "	28.8	28.9
5	0.5 " "	29.0	29.0
10	0 5 " "	29.5	29.5
20	0.5 " "	28.3	28.3
10	0 05 " "	25.3	27.4
10	0.1 " "	27.9	27.6
10	0.2 " "	28.0	27.6
10	0.5 " "	28.6	28.2
10	1.0 " "	27.9	28.0
10	0.1 " CH₂COOH	27.5	27.4
10	0.2 " "	28.0	27.4
10	0.5 " "	28.0	26.8
10	1.0 " "	28 6	27.0

TABLE II

Effect of Gelatin on Stability of p-Aminobenzoic Acid and p-Aminobenzoylglutamic Acid to Reduction by Zinc

Compound	Time of reduction	Before reduction	After reduction	
			No gelatin	With gelatin
	min.	*γ per ml.*	*γ per ml.*	*γ per ml.*
p-Aminobenzoic acid	10	100	79.0	100
	60	100	45.6	98
p-Aminobenzoylglutamic acid	10	103	92.3	103
	60	103	81.5	102

sunlight), and in ordinary glassware in direct sunlight. The results are presented in Table III. It is quite apparent that the p-aminobenzoyl-glutamic acid diazo compound is unstable to direct sunlight. In ordinary

laboratory light (no direct sunlight) the diazotization product appears to be stable. However, if there is a possibility of direct sunlight, the diazotization and coupling should be carried out in non-actinic glassware.

Stability of Pteroylglutamic Acid to Light—Since it has been shown that pteroylglutamic acid is decomposed by light to yield p-aminobenzoylglutamic acid (2), the analytical procedures for this compound should be carried out in the absence of direct or indirect sunlight.

Correlation with Biological Activity—In certain synthetic products the chemical method gives higher values than does microbiological assay. This is interpreted to mean that the isomeric 7-pteridyl compound is also reduced to yield an aromatic amine. The extent of reduction of the isomeric compound has not been studied on a pure product. However, a comparison of the chemical values with the values obtained on microbiological assay serves to indicate the proportion of active isomer present.

TABLE III

Stability of p-Aminobenzoylglutamic Acid Diazo Compound to Light

Conditions of lighting	p-Aminobenzoylglutamic acid
	per cent
Red flask	99.6
Laboratory light	98.9
Direct sunlight.	56.9

When only the active isomer is formed, there is complete correlation between the chemical and biological activity.

Interfering Compounds—Any compound that will give rise to an aromatic amine on reduction, which will develop a color in the Bratton and Marshall determination, will interfere. The distribution of such compounds in natural products is unknown.

Interfering compounds in the crude products arising from synthesis are the isomeric compound mentioned above and oxidized amines. The oxidized amines are removed by preliminary purification of the active compound.

Utility of Method—The method is satisfactory with concentrates derived from natural sources when the content of the active compound is 5 per cent or greater. The method is entirely satisfactory for determining the potency of crude products derived from various synthetic reactions when only the naturally occurring isomer is present.

When the method is used for the various derivatives or analogues, the desired factor is obtained from the following ratio, (molecular weight of analogue)/(molecular weight of p-aminobenzoic acid).

DISCUSSION

On the basis that the reduction proceeds essentially to completion, the accuracy of the method is determined by the accuracy of the Bratton and Marshall procedure, which is ±2 per cent.

As the sensitivity of the colorimetric procedure is not great, the chemical method is of no value in determining the pteroylglutamic acid content of natural materials unless the active compound is present at a concentration sufficient to give a readable color.

Zinc dust containing from 1 to 3 per cent copper has been used as the reducing agent. The reduction is somewhat more vigorous but offers no particular advantages over zinc dust alone. Zinc amalgam (containing from 0.1 to 4.3 per cent zinc) is a satisfactory reducing agent.[2] In this modification the reduction is carried out for 30 minutes on a shaking machine. The values obtained with zinc amalgam are similar to those obtained with zinc dust when gelatin is present but somewhat higher than the values obtained with zinc dust alone.

Only approximate values could be obtained for pteroic acid by the chemical method because of the extreme insolubility of this compound in acid solution. This is a specific rather than a general property of this group of compounds.

SUMMARY

A chemical method for the determination of pteroylglutamic acid and related compounds is outlined. The method is based on the fact that these compounds are cleaved by reduction in an acid solution to yield a pteridine and an aromatic amine. The amount of aromatic amine formed during the reduction is determined by the method of Bratton and Marshall and is used as a measure of the pteroyl derivative present.

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AN IMPROVED METHOD FOR THE DETERMINATION OF TRYPTOPHANE WITH p-DIMETHYLAMINO-BENZALDEHYDE

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The modifications proposed in this paper permit the original Bates method (1) to be generally applied to the assay of tryptophane in proteins so that a simple, rapid, and accurate method for determining this amino acid is achieved. This is accomplished by developing the standard color in the presence of gelatin and by making certain changes in the concentrations of the reagents so that interference from cystine is eliminated.

EXPERIMENTAL

Influence of Gelatin on Production of Blue Color[1]—When pure tryptophane was treated by the short procedure of Bates, more color was produced and fading was less rapid in the presence of gelatin than when no gelatin was added.

Table I shows that maximum color was produced in the presence of gelatin when the time of development was between 10 and 90 minutes. No greater color intensity could be produced when the $NaNO_3$ was increased from 0.2 cc. of 1.5 per cent to 0.2 cc. of 5 per cent solution.

In the absence of gelatin, the color does not reach maximum intensity regardless of time, probably because of the destruction of tryptophane in strong HCl. Table I indicates a color intensity 20 per cent greater for the standard to which gelatin was added. Accordingly, tryptophane values obtained by reading from a standard curve prepared without gelatin, as is the usual custom, are too high.

The data were obtained as follows: 2 cc. of a solution containing 0.3 mg. of *l*-tryptophane (Merck) were added to each of twenty 100 cc. volumetric flasks. 35 mg. of gelatin[2] were added to each of ten of these and dissolved by warming the flasks. Color was developed by the Bates method with the modifications described later. Varying lengths of time

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[1] Sullivan *et al.* (2) mentioned the instability of free tryptophane in acid solution and suggested protection by means of an amino acid mixture such as occurs in casein. Doty (3) added gelatin to the assay mixture without indicating a reason.

[2] Wilson's pure food gelatin, porcine type. This product gave no color test for tryptophane when tested by the Bates procedure.

were allowed for color development, after which the solutions were made to volume with 50 per cent alcohol as suggested by Dr. Bates.[3] The light transmission was observed in a Lumetron colorimeter, with a No. M550 filter (maximum transmission 550 mμ).

Shaw and McFarlane (4) have stated that tryptophane combined in the protein molecule gives more color with p-dimethylaminobenzaldehyde than when free and that a source of error lies in the use of free tryptophane as a standard. The evidence suggests that free tryptophane may be employed if protected by a protein or a mixture of amino acids.

TABLE I

Effect of Gelatin on Color Formation in p-Dimethylaminobenzaldehyde-Tryptophane Reaction

Time for color development	Transmission	
	Gelatin, 35 mg.	No gelatin
min.	*per cent*	*per cent*
5	60.0	62.3
10	46.8	57.0
15	46.5	54.6
20	46.7	54.1
25	46.5	54.5
30	46.6	54.2
60	46.9	54.9
90	47.0	62.6
120	47.6	67.2
150	49.0	69.1

Influence of Cystine on Determination of Tryptophane in Proteins—Originally this method was applied only to casein by Bates who dissolved the sample in 0.1 N NH$_4$OH or NaOH. Since some proteins cannot be dissolved in this manner,. it was necessary to employ alkaline hydrolysis.

In our first trials 10 to 20 mg. of protein and 35 mg. of gelatin were boiled with 2 cc. of 5 N NaOH for 2 hours. After cooling the hydrolysate to room temperature, color was developed by the method of Bates with 0.2 cc. of 1.25 per cent NaNO$_3$, and tryptophane concentrations were read from the standard curve described below.

Under such conditions, the tryptophane content of Labco casein and of crystalline bovine serum albumin (Armour) was found to be 1.32 and 0.30 per cent respectively. While this value for casein is acceptable, the value for serum albumin is lower than that reported by Brand *et al.* (5) who used the spectrophotometric determination of the isolated mercury compound

[3] Private communication, 1938.

of tryptophane. When a longer time was allowed for color development, the serum albumin value was not increased. However, when hydrolysis was performed with 1 N alkali and the concentration of the $NaNO_3$ reagent was increased to 2 per cent, an average value of 0.57 per cent was obtained, which agreed with the value 0.58 per cent reported by Brand *et al.*

The amino acid composition of bovine albumin as published by Brand *et al.* (5) indicates the presence of an unusually high proportion of cystine. Casein, which does not retard color formation under any condition, yields a very small proportion of cystine.

When 5 N NaOH and 1.25 per cent $NaNO_3$ were used, it was found (Table II) that cystine added to casein after hydrolysis in the proportion present in bovine albumin had no effect, but when added prior to hydrolysis, it decreased color formation. Cysteine, however, decreased color formation when added after hydrolysis.

TABLE II

*Effect of Cystine and Cysteine on Color Development with Labco Casein**

20 mg. of casein were hydrolyzed for 2 hours with 5 N NaOH. Color was developed with 0.2 cc. of 1.25 per cent $NaNO_3$.

Substance added	Time of addition	Tryptophane found
		per cent
None		1.30
Cystine, 1.2 mg.	After hydrolysis	1.28
" 1.2 "	Before "	0.53
Cysteine, 1.2 mg.	After "	0.62

* The values are averages of two trials.

Procedure for Assay—10 to 20 mg. of protein (containing 0.1 to 0.4 mg. of tryptophane) and 35 mg. of gelatin are refluxed with 2 cc. of 1 N NaOH for 2 hours. After the mixture has cooled to room temperature, to each sample are added with agitation 0.5 cc. of 2.5 per cent *p*-dimethylamino-benzaldehyde in 10 per cent H_2SO_4, 0.2 cc. of 2 per cent $NaNO_3$, and 28 cc. of concentrated HCl. The solutions are allowed to stand for 30 minutes, then diluted with 50 per cent alcohol to 100 cc., and read in the colorimeter. A reagent blank, containing all of the above reagents except the unknown and treated as described, is set at 100. From the per cent transmission, tryptophane concentration is read from a standard curve. This curve is established by means of a series of flasks containing 0 to 0.6 mg. of tryptophane and 35 mg. of gelatin treated as described. The logarithm of the per cent transmission plotted against concentration gives a straight line.

The results of tests of the recovery of tryptophane added to proteins prior to hydrolysis are shown in Table III.

Proteins Assayed—Nitrogen determinations are reported on the ash-, water-, and fat-free basis.

Casein. Labco casein, Lot 10588; nitrogen content 15.6 per cent.

TABLE III

*Recovery of Tryptophane Added to Proteins Prior to Hydrolysis**

The proteins were hydrolyzed 2 hours with 1 N NaOH   Color was developed with 0.2 cc. of 2 per cent NaNO₃.   0.100 mg of tryptophane added to the proteins

Protein	Tryptophane present	Tryptophane found	Recovery of tryptophane added
	mg	*mg*	*per cent*
Casein	0 247	0 348	100 3
Bovine albumin	0 116	0.215	99 5

* The figures are averages of four trials

TABLE IV

*Tryptophane Content of Animal Proteins**

Protein	Tryptophane		Bibliographic reference No ·
	Authors' data	Literature	
	per cent	*per cent*	
Casein	1 32	1 3,† 1 1,‡ 1 54,§ 1 8,§ 1 24†	(7 11)
Beef muscle.	1.26	1 24,‡ 1 35,§ 1 2§	(8, 12, 10)
Lactalbumin	2.35	2 1,† 2.77,§ 2 3,§ 1.9,‖ 1 81‖	(7, 9, 10, 13, 14)
Beef fibrin	4 0	3 5, 3 51,† 3 7,§ 3 56§	(15 17, 9)
Bovine albumin	0.57	0.58¶	(5)
Beef serum.. .	1 55	1.7§	(10)
Ovalbumin	1.29	1.22,† 1.28,** 1.4§	(11, 18, 10)
Wheat gluten	0.91	0.93,§ 1 0§	(9, 10)
Soy flour .	1.22	1 0, 1 5§	(15, 17)
" protein	1 52	1 5§ .	(17)

* Reported on an ash-, water-, fat-free basis.   The values are averages of four determinations.

† Moisture-, ash-free basis

‡ Moisture-free basis.

§ 16 per cent nitrogen basis.

‖ 14.2 per cent total nitrogen.

¶ 16.07 per cent total nitrogen.

** 15.12 per cent total nitrogen.

Beef muscle. Beef shank, vacuum-dried and defatted with benzene; nitrogen content 16.2 per cent.

Bovine albumin. ·Crystalline serum albumin, Armour, Lot 45; nitrogen content 15.8 per cent.

Fibrin, lactalbumin, and beef serum. The same samples as previously reported by this laboratory (6).

Ovalbumin. A crystalline product obtainéd through the courtesy of Dr. A. G. Cole, College of Medicine, University of Illinois; nitrogen content 14.4 per cent.

Wheat gluten. From the Pure Gluten Food ·Company, New York; nitrogen content 14.2 per cent.

Soy flour (solvent-extracted) and soy protein (α-). Courtesy of The Glidden Company, Chicago, nitrogen content 9.4 and 15.4 per cent respectively.

Tryptophane values obtained by this method for the preparations listed above are shown in Table IV and the results compared with those reported by other workers. The agreement with values from the literature obtained by reliable procedures is good.

DISCUSSION

The Bates method for tryptophane, as modified herein, gives a rapid, accurate procedure for the colorimetric determination of this amino acid in proteins. Routinely, six samples can be assayed in 4 hours.

The original method was applied only to proteins readily soluble in 0.1 N alkali. The method reported here is applicable to all ordinary proteins examined in our laboratory, including those which are not soluble in dilute alkali.

Paradoxically, the original Bates method gives values for some proteins which are too high and for others too low. The high values appear to result from the instability of the tryptophane standard in acid solution. The low values are due to the effect of reducing substances produced during hydrolysis from excessive cystine in the sample. These effects are eliminated by use of the present procedure.

SUMMARY

A simple, rapid method for the determination of tryptophane in proteins is described, involving the use of a modification of the Bates procedure.

The tryptophane content of several proteins as determined by this method is reported.

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THE EFFECT OF SUCCINIC DEHYDROGENASE ON DEUTERIUM-LABELED SUCCINIC ACID IN THE ABSENCE OF HYDROGEN ACCEPTORS

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It is now well established that the oxidation of metabolites by living cells involves the passage of hydrogen or electrons or both along a series of oxidation-reduction systems. Oxygen enters this chain of reactions only at the terminal steps involving the cytochrome oxidase system. The mechanisms by which biological oxidations are carried out have been recently reviewed by Potter (1, 2) and by Ball (3).

The fundamental type of reaction involved in the respiration chain is shown by the relationship

$$SH_2 \quad + \quad Acc \quad \xrightarrow{\text{dehydrogenase}} \quad P \quad + \quad AccH_2$$

| Oxidizable substrate | Hydrogen acceptor | Oxidized product | Reduced acceptor |
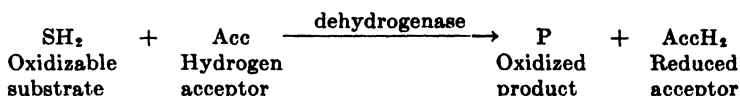
The oxidation of a substrate, thus, is accomplished by the removal of hydrogen atoms which pass to a hydrogen acceptor. This reaction proceeds only in the presence of enzymes which are usually specific for the substrate and sometimes specific for the acceptor, such enzymes being classed as dehydrogenases.

It is the purpose of this work to present data bearing on the problem of the mechanism of biological hydrogen transfer. For this study succinic acid dehydrogenase, an extensively studied enzyme typical of the dehydrogenase group, was selected, and use was made of deuterium to label the carbon-bound hydrogen atoms of succinic acid.

The reaction catalyzed by succinic acid dehydrogenase is

$$HOOC-CH_2-CH_2-COOH + Acc \rightarrow HOOC-CH=CH-COOH + AccH_2$$
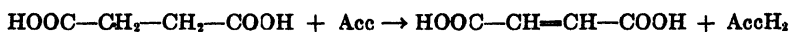
Methylene blue has most frequently served as the hydrogen acceptor for succinic acid oxidations *in vitro*. In order to study any direct transfer of hydrogen atoms in the above reactions, it would be desirable to label the

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hydrogen atoms of succinic acid, and then, after incubation with an acceptor such as methylene blue, to isolate the acceptor and determine its deuterium content. This is not feasible, however, since the removable hydrogens of quinoid dyes such as methylene blue are dissociable, and would not be expected to stay on the dye. The experiments were, therefore, planned and carried out in such a way as to avoid the oxidation of succinic acid. The exchange of deuterium and hydrogen between the succinate and water and the influence of the enzyme upon this exchange were determined. In most cases no acceptor was added, but in a few experiments the effect of methylene blue on the hydrogen exchange was studied.

Erlenmeyer, Schoenauer, and Süllmann (4) prepared deuterosuccinic acid and incubated it with succinic dehydrogenase in the presence of methylene blue. They found that the fumaric acid that was formed as well as the unchanged succinic acid contained a higher hydrogen to deuterium ratio than the initial succinic acid, and suggested that hydrogen exchange occurred from the leucomethylene blue.

Methods

Preparation of Succinic Acid Dehydrogenase—The preparation of succinic acid dehydrogenase was based on the procedures of Ogston and Green (5) and Schneider and Potter (6). Rabbit kidneys were homogenized with a few ml. of distilled water in a test-tube homogenizer (Potter and Elvehjem (7)), care being taken to keep the temperature low throughout the process. The homogenate was diluted with distilled water to a volume equivalent to 3 times the weight of the kidneys, and was allowed to stand in the refrigerator for 30 minutes. It was then centrifuged at high speed for 30 minutes. The enzyme, contained in the supernatant liquid, was stored in a solid CO_2 chamber until used, which usually occurred within 3 days of its preparation.

The activity of the enzyme preparation was measured by the Thunberg technique. The main compartment of the Thunberg tubes contained 0.5 ml. of the enzyme preparation, 0.2 ml. of 0.1 M phosphate buffer, pH 7, and 0.15 ml. of 20 per cent sodium succinate or distilled water. The side arms contained 0.1 ml. of 0.00033 per cent methylene blue. After evacuation and temperature equilibrium the methylene blue and enzyme were mixed, and the time necessary to reduce the methylene blue was noted. The enzyme activity was then calculated and expressed as the mg. of succinic acid oxidized to fumaric acid per hour per ml. of enzyme preparation.[1]

$$\text{Activity} = \text{mg. MB reduced} \times \frac{\text{mol. wt. of SA}}{\text{mol. wt. of MB}} \times \frac{60 \text{ min}}{\text{reduction time}} \times \frac{1}{\text{ml. of enzyme}}$$

[1] MB = methylene blue; SA = succinic acid.

This calculation facilitated comparison between the rate of succinate oxidation and the rate of hydrogen exchange.

Incubations—Unless otherwise indicated, the incubations were carried out anaerobically in small bottles of about 35 ml. capacity, filled, and sealed so as to exclude entry of oxygen. Known amounts of deuterosuccinic acid were dissolved in water, neutralized to pH 7, and the desired aliquot pipetted into bottles. The amount of succinic acid incubated was always between 100 and 200 mg. 3 to 6 ml. of 0.1 M phosphate buffer (pH 7) was added to the bottle. The desired amount of enzyme was then added along with any special reagents such as methylene blue. The bottles were completely filled with distilled water and were sealed off by means of a screw top. The bottles were placed in a mechanical agitator at room temperature and gentle agitation continued throughout the incubation. The reaction was stopped by precipitating the proteins with phosphomolybdic acid, after which the succinic acid was isolated.

Isolation of Succinic Acid—Succinic acid was isolated from the phosphomolybdic acid filtrates of the incubation mixtures by a slight modification of the method of Goepfert (8). This method involves the oxidation of impurities with permanganate (Wood *et al.* (9)), extraction with diethyl ether in a continuous extractor, and precipitation of the succinate as the silver salt at pH 5.6.

Determination of Deuterium—The silver succinate was analyzed for its deuterium content by methods described by Rittenberg and Schoenheimer (10) and Keston, Rittenberg, and Schoenheimer (11). A weighed amount of silver succinate (usually between 300 and 500 mg.) was oxidized by dry, hydrogen-free oxygen in a combustion furnace, and the water collected in a trap submerged in a dry ice bath. The combustion water was purified in a train of U-tubes by an alkaline permanganate oxidation, a chromic acid oxidation, and a redistillation. The amount of water resulting from the oxidation of the silver succinate was determined from the loss of weight of the first tube as the water was distilled off.

Preparation of Deuterosuccinic Acid—Deuterosuccinic acid was prepared by dissolving ethyl fumarate in cyclohexane and saturating it with deuterium in the presence of platinum oxide catalyst in the apparatus described by Rittenberg and Schoenheimer (10). The cyclohexane was distilled off at atmospheric pressure, and the ethyl deuterosuccinate was collected at 210–220°. This was saponified with an excess of sodium hydroxide, and the sodium salt of deuterosuccinic acid was purified by the permanganate oxidation and ether extraction process already described. The combustion water of the deuterosuccinate contained 34 per cent D_2O, corresponding to 32.3 atoms per cent deuterium.

. *Results*

The results of incubating deuterosuccinic acid with kidney succinic acid dehydrogenase preparations are shown in Table I. A definite exchange of hydrogen atoms of the solution with the deuterium atoms of the deuterosuccinate in the absence of enzyme was found in the controls (Experiments 1 and 4). However, in the presence of the enzyme preparation, a significant increase in the exchange was apparent in all experiments. The presence of methylene blue had no influence on the rate of exchange. The enzyme activity and incubation time of the experiments of Series I was such as to bring about the oxidation of 9.5 mg. of succinic acid. This is 5 per cent of the total succinic acid present. It is seen that

TABLE I

Exchange of Hydrogen Ions with Deuterosuccinate

Series No.	Experiment No.	Succinic acid incubated	Enzyme used	Activity of enzyme	Methylene blue	Incubation period	Succinic acid recovered	D₂O in combustion water	Per cent exchanged*
		mg.	*ml.*	*units per ml.*	*mg.*	*hrs.*	*gm.*	*per cent*	
I	1	199	0		0	4	110	32 8	3 5
"	2	199	15	0 158	0	4	160	30 2	11 2
"	3	199	15	0 158	11	4	125	30 9	9 1
II	4	185	0	0 135	0	25	150	32.3	5 0
"	5	185	25	0.135	0	4	137	28 3	16 8
"	6	185	25	0 135	0	8	143	26.7	21 5
"	7	185	25	0 135	0	25	165	16 9	50 3
"	8	185	25	0 135	11	25	157	17 8	47 7
III	9	118	25	0 237	0	31	79	3 5	89 7

* Per cent exchanged $= \dfrac{34 - \% \ D_2O \ \text{in combustion water}}{34} \times 100.$

the loss of deuterium from the succinic acid was also of this order of magnitude, after the exchange in the absence of enzyme is subtracted.

In Series II, the amount of enzyme and the incubation time were increased in order to accentuate the exchange. The exchange was increased by this procedure up to 50 per cent of the maximum possible with 25 ml. of enzyme and an incubation time of 25 hours. In Experiment 9 in which 118 mg. of deuterosuccinic acid were incubated for 31 hours with 25 ml. of an enzyme having an activity of 0.237, 90 per cent of the deuterium atoms of deuterosuccinic acid was replaced by hydrogen atoms from the solution.

The rate of hydrogen exchange can be roughly compared with the rate at which succinic acid would be oxidized in the presence of an acceptor

by calculating the latter from the enzyme activity and the incubation time.

Succinate oxidizable = enzyme activity × ml. of enzyme × incubation time

Such a calculation depends upon the assumption that the activity of the enzyme is not significantly changed during the incubation period. This assumption was verified by testing for the rate of methylene blue reduction of samples incubated under conditions identical with those prevailing in the experimental incubations. Usually the enzyme activity was slightly greater at the end of 24 hours than at the initiation of the experiment. The results of making this comparison are shown in Table II, where it is evident that the exchange of hydrogen and deuterium on the deutero-

TABLE II

Comparison between Per Cent Deuterium Exchange and Theoretical Per Cent of Succinate Oxidizible during Incubation Period

Series No.	Experiment No	Per cent deuterium exchange of experiment (minus blank exchange)	Per cent succinic acid theoretically oxidizable*
I	2	7.7	4.8
"	3	5.6	4.8
II	5	16.0	7.3
"	6	19 9	14.6
"	7	45.3	45.6
"	8	42.7	45.6
III	9	89.7	156.0

* Calculated from the relation, enzyme activity × ml. of enzyme × incubation time.

succinate proceeded at essentially the same rate as the oxidation of succinate would have progressed had methylene blue been present.

DISCUSSION

From the data presented it becomes evident that the α- and α'-hydrogen atoms of succinic acid will exchange with hydrogen ions of the solution under the influence of an enzyme contained in kidney preparations. There are several explanations which might account for this effect. The most likely possibility is the presence of succinic acid dehydrogenase which catalyzes the oxidation of succinic acid to fumaric acid. Results in Table II show that the percentage of exchange noted is about the same as the percentage of succinic acid which could theoretically have been oxidized in the indicated period. This agreement supports the hypothesis, which we hold, that the same enzyme, succinic acid dehydrogenase, is responsible

for both of the following reactions, oxidation of succinate (1) and hydrogen exchange (2).

$$HOOC—CH_2—CH_2—COOH \ + \ MB \ \xrightarrow[\text{dehydrogenase}]{\text{succinic}} \qquad (1)$$

$$HOOC—CH{=}CH—COOH \ + \ MBH_2$$

$$\underset{\quad D \quad \; D}{HOOC—CH—CH—COOH} \ + \ 2H^+ \ \xrightarrow[\text{dehydrogenase}]{\text{succinic}} \qquad (2)$$

$$\underset{\qquad H \quad \; H}{HOOC—CH—CH—COOH} \ + \ 2D^+$$
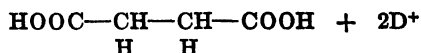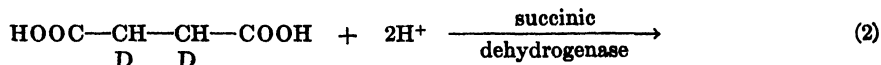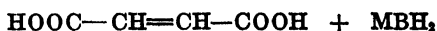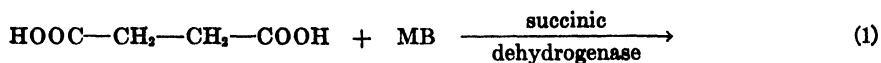
While succinic acid dehydrogenase may thus be considered the most likely cause of hydrogen exchange, other possibilities should be considered. Another unrelated enzyme present in the preparation might, for example, have catalyzed the exchange.

An exchange of hydrogen atoms of a substrate with hydrogen ions of the environment is accompanied, of course, by a dissociation of hydrogen atoms into hydrogen ions and electrons. It thus would appear that hydrogen transfer in biological oxidations, even below the point at which the iron-containing cytochrome system comes into play, involves this dissociation of substrate hydrogen atoms.

This interpretation is weakened by the fact that a crude enzyme preparation was used in these experiments and may have contained a naturally occurring intermediary acceptor. Such an acceptor might exchange its reducible hydrogens with hydrogen ions, and, being in equilibrium with the enzyme-succinate complex, could thus lower the deuterium content of the isolated succinic acid. This would be in accord with the conclusion of Erlenmeyer *et al.* (4) who postulated an exchange between hydrogen ions and the hydrogen atoms of leucomethylene blue produced in the oxidation of deuterosuccinic acid to fumarate. Similarly the enzyme might itself contain a reducible prosthetic group which could exchange hydrogen with the hydrogen ions of the environment. It is not possible from the data to draw conclusions as to whether the observed exchange occurs from the enzyme-substrate complex, from the enzyme prosthetic group, or from a hydrogen acceptor in the crude enzyme preparation. It is of interest to note in this connection that methylene blue, either in large amounts or in traces, did not influence the speed of the hydrogen exchange. This would support the interpretation that the mechanism of such an exchange depends upon the enzyme only, and is independent of any free acceptor.

The data and interpretation given in this paper are in accord with the theory of the mechanism of succinic acid dehydrogenase action advanced

by Potter and DuBois (12). These workers suggest that the active center of succinic acid dehydrogenase consists of a sulfhydryl group located between the two carboxyl affinity points, *i.e.* between the enzyme groups that bind the carboxyl groups of succinic acid. The enzyme was visualized as functioning by oscillating between the normal sulfhydryl form and its free radical, with electrons passing to the prosthetic group of the enzyme and hydrogen ions passing into the solution.

SUMMARY

By use of deuterium to trace the reaction it has been shown that an enzyme will anaerobically catalyze the exchange of the α- and α'-hydrogen atoms of succinic acid with hydrogen ions from the solution. Rate studies suggest that the same enzyme, succinic acid dehydrogenase, catalyzes this hydrogen exchange as well as the dehydrogenation of succinic acid to form fumaric acid.

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STUDIES WITH BACTERIAL SUCROSE PHOSPHORYLASE

I. THE MECHANISM OF ACTION OF SUCROSE PHOSPHORYLASE AS A GLUCOSE-TRANSFERRING ENZYME (TRANSGLUCOSIDASE)*

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It has been shown that sucrose phosphorylase preparations from *Pseudomonas saccharophila* catalyze the reversible reaction between glucose-1-phosphate and certain ketose sugars. This reaction results in the formation of sucrose and analogous disaccharides together with the liberation of inorganic phosphate (1–5). In the experiments reported here, it was observed that when glucose-1-phosphate and radioactive inorganic phosphate are added to enzyme preparations in the absence of ketose sugars, a rapid interchange of phosphate occurs between the organic and inorganic fractions.

This indicated that the enzyme can liberate inorganic phosphate from glucose-1-phosphate without the production of an equivalent amount of glucose. The following reaction was postulated,

$$\text{Glucose-1-phosphate} + \text{enzyme} \rightleftharpoons \text{glucose-enzyme} + \text{phosphate} \qquad (1)$$
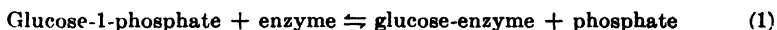
The glucose-enzyme bond presumably retains the energy of the phosphoric ester linkage. This observation suggested that an analogous reaction would occur between the enzyme and sucrose, with fructose being liberated instead of phosphate,

$$\text{Glucose-1-fructoside} + \text{enzyme} \rightleftharpoons \text{glucose-enzyme} + \text{fructose} \qquad (2)$$
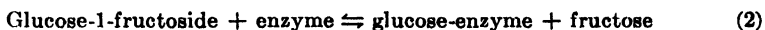
Reactions (1) and (2) can account for the ability of the enzyme to substitute a glycosidic linkage for a phosphoric ester bond.

The postulated mechanism is strongly supported by experiments in which virtually phosphate-free enzyme preparations were found capable of synthesizing the sucrose analogue, glucosidosorboside, directly from sucrose and sorbose,

$$\text{Glucose-1-fructoside} + \text{sorbose} \rightleftharpoons \text{glucose-1-sorboside} + \text{fructose} \qquad (3)$$

In like manner, sucrose was produced from its synthetic analogue, glucosidoketoxyloside and fructose,
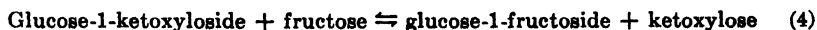
$$\text{Glucose-1-ketoxyloside} + \text{fructose} \rightleftharpoons \text{glucose-1-fructoside} + \text{ketoxylose} \qquad (4)$$

These results show that sucrose phosphorylase not only catalyzes the substitution of glycosidic linkages for a phosphate linkage but also causes an exchange between equivalent glycosidic linkages.

<center>EXPERIMENTAL</center>

<center>*Reaction between Glucose-1-phosphate and Enzyme*</center>

The sucrose phosphorylase preparation was obtained by methods previously described, except that it was reprecipitated six times with 0.63 saturated ammonium sulfate (6, 2). A dilute solution of the enzyme was incubated at 30° with 0.1 M potassium glucose-1-phosphate and 0.033 M potassium phosphate containing P^{32}. Parallel experiments were conducted in which fructose and glucose were added separately to the above mixture.

<center>TABLE I</center>

<center>*Exchange of P^{32} between Inorganic Phosphate and Glucose-1-phosphate*</center>

Experiment No	Reaction mixture	Radioactivity found in fractions, counts per min per micromole after 60 min. at 30°	
		Inorganic phosphate	Glucose-1-phosphate
1	0.1 M glucose-1-phosphate + 0.033 M inorganic phosphate	1098 (±40)	0 (±1)
2	Same as (1) but with enzyme preparation	859 (±40)	119 (±3)
3	Same as (2) but with 0.06 M fructose	886 (±40)	99 (±3)
4	Same as (2) but with 0.12 M glucose	1096 (±40)	7 (±1)

Fructose was used since it is known to participate in a reversible reaction involving glucose-1-phosphate and inorganic phosphate, while glucose has been previously shown to have a strong inhibitory action on sucrose phosphorylase (6). After incubation, the enzyme was inactivated by boiling, and the inorganic phosphate was precipitated as the barium salt, redissolved with acid, and reprecipitated for analysis. The remaining traces of radioactive inorganic phosphate were removed from the solution of glucose-1-phosphate by repeated additions of inorganic phosphate and its removal with barium. Chemical analyses were then made on the inorganic and organic fractions and radioactivity was determined with a Geiger counter. A control experiment, in which glucose-1-phosphate and radioactive inorganic phosphate were incubated together in the absence of enzyme, showed that no non-enzymatic exchange occurs between these compounds.

The results of the experiment with sucrose phosphorylase are presented n Table I. No appreciable formation of free glucose from glucose-

1-phosphate could be detected during the course of the experiment. Sucrose was formed in the presence of fructose, but the rate of its formation was not determined.

The experiment shows that P^{32} appeared rapidly in the glucose-1-phosphate fraction, even though a dilute enzyme preparation was used. In the presence of fructose, less P^{32} was found in glucose-1-phosphate. This is due, at least in part, to a dilution of the radioactive phosphate with inactive phosphate liberated as a result of the synthesis of sucrose. The principal reason, however, must be the competition of fructose with phosphate for combination with glucose.

The presence of glucose almost completely stops the exchange of P^{32}. This is consistent with the observation that glucose inhibits phosphorylase activity and supports the view that glucose competes with glucose-1-phosphate for combination with the enzyme.

Interconversion of Disaccharides in Absence of Phosphate

In order to show that the enzyme can liberate fructose from sucrose without the formation of either free glucose or glucose-1-phosphate, an experiment was devised in which its ability to produce glucosidosorboside from sucrose in the absence of glucose-1-phosphate was tested. To prevent the accumulation of glucose-1-phosphate in the reaction mixtures, the enzyme preparations were rendered virtually free of inorganic phosphate. The initial steps of obtaining the enzyme from dried cells of *Pseudomonas saccharophila* were identical with the previously described method. After three reprecipitations of the enzyme with ammonium sulfate from phosphate buffer, the protein precipitate was redissolved in phosphate-free 0.05 M citrate buffer at pH 6.7 containing 0.01 M KCl. The enzyme was precipitated with recrystallized ammonium sulfate at 0.63 saturation. After three further reprecipitations from citrate buffer, carried out over a period of 2 days, the enzyme was redissolved in the same buffer mixture and used in the experiments. Such preparations were found to retain strong phosphorylase activity. The rate of glucosidosorboside formation is low, regardless of whether glucose-1-phosphate or sucrose is used as substrate for the enzyme. For this reason, the experiments were of fairly long duration and a relatively high concentration of enzyme had to be used. Enzyme preparations from ±1 gm. of dry cells were used per 20 ml. of final experimental solutions. As a result, an appreciable amount of reducing sugar was formed from sucrose in the course of the experiments. This phenomenon has been noted previously and is ascribable to traces of invertase in the preparation. Less than 10^{-5} M phosphate was found to be present in the final reaction mixtures.

Sucrose was estimated from the reducing value obtained on hydrolysis

with invertase. Neither glucosidosorboside nor glucosidoketoxyloside is attacked to an appreciable extent by invertase. These compounds, therefore, could be estimated in phosphate-free mixtures from the difference between reducing values obtained upon hydrolysis with invertase and hydrolysis with 0.2 N HCl for 5 minutes at 100°. In experiments in which phosphate was added to the reaction mixture, the glucose-1-phosphate which was produced was measured as inorganic phosphate released on 7 minutes hydrolysis with 0.1 N HCl. The glucosidosorboside produced in experiments conducted in the presence of phosphate was estimated from the difference between initial and final reducing sugar values obtained

TABLE II

Production of Glucosidosorboside from Sucrose

Experiment No	Additions to enzyme preparation (different preparations used for Experiments 1 and 2)	Time of incubation at 30°	Total sucrose disappearing	Sucrose phosphorolyzed	Glucosidosorboside produced
		hrs.	mg. per ml.	mg. per ml.	mg. per ml.
1, a	0.05 M sucrose	2	0.3 (±0 3)	0	0
b	0 05 " " 0.14 M sorbose	2	2.9 (±0.3)	0	2 7 (±0.3)
c	0.05 " " 0.14 " "	4	4.9 (±0 3)	0	4 4 (±0.3)
d	0 05 " " 0 14 " " 7 × 10⁻⁴ M Na₂HPO₄	2	3 0 (±0 3)	0 2	2.2 (±0.3)
e	0.05 M sucrose, 0 14 M sorbose, 7 × 10⁻³ M Na₂HPO₄	2	3 7 (±0 3)	2 4	0 6 (±0.3)
f	0 05 M sucrose, 0.14 M sorbose, 7 × 10⁻³ M Na₂HPO₄	4	5.5 (±0.3)	2 3	2.5 (±0.3)
2, a	0.06 M sucrose, 0 11 M sorbose	3	4.9 (±0 3)	0	4.6 (±0.3)
b	0 06 " " 0.11 " " 0.12 M glucose	3	0 6 (±0.3)	0	0.3 (±0.3)

on hydrolysis with invertase and corrected for the decrease in reducing value due to the formation of glucose-1-phosphate.

When sucrose and sorbose were added to the enzyme preparations in the absence of phosphate, sucrose disappeared and glucosidosorboside was formed (see Experiment 1, Table II). Since at equilibrium considerably less of the latter than of the former would be expected (3), the rate of transformation must decrease rapidly in the course of the experiment. The initial rate of conversion must, therefore, be greater than that which was observed for the first 2 hours.

The initial rate of glucosidosorboside formation by the same enzyme preparation could be measured with a fair degree of accuracy when glucose-1-phosphate was used as substrate in place of sucrose, by determining the rate of the evolution of inorganic phosphate. In the presence of 0.14 M

sorbose, and 0.025 M glucose-1-phosphate, the initial rate of disaccharide synthesis was found to be 3.24 mg. per 2 hours per ml. at 30°. At this concentration, glucose-1-phosphate is not limiting to the rate of reaction. When the concentration of glucose-1-phosphate was reduced to 0.002 M, the initial rate was found to be 2.72 mg. per 2 hours per ml., while at 0.001 M concentration, the rate was 2.14 mg. per 2 hours. It can be roughly computed from these data that with 10^{-5} M glucose-1-phosphate the rate would be in the neighborhood of 0.06 mg. of glusosidosorboside formed per 2 hours per ml. Since the maximum limit of inorganic phosphate present as impurity in the "phosphate-free" reaction mixtures was found to be 10^{-5} M, this would be the maximum concentration of glucose-1-phosphate which could be attained. Since the observed rate of glucosidosorboside formation from sucrose· approaches the maximum rate of synthesis from glucose-1-phosphate, it is clear that the interconversion of disaccharides cannot depend on a preliminary phosphorolysis and a subsequent utilization of the accumulated glucose-1-phosphate.

This view is further supported by the fact that the addition of phosphate to reaction mixtures does not increase the rate of interconversion of disaccharides but decreases it. This inhibitory effect of phosphate is entirely in accord with the postulated hypothesis for the mechanism of enzyme action. Not only the phosphate, itself, but also the fructose liberated in the phosphorolysis must compete with sorbose for the enzyme-glucose complex.

The hypothesis also requires that glucose will inhibit the conversion of sucrose to glucosidosorboside by competing with the glucose portion of the sucrose molecule for a position on the enzyme. The competition between glucose and sucrose has already been shown in studies of the phosphorolytic breakdown of sucrose (6). That the rate of interconversion of the disaccharides is indeed decreased by the addition of glucose is demonstrated by Experiment 2 (Table II).

The quantitative discrepancy between the sucrose disappearance and glucosidosorboside formation in both Experiments 1 and 2 (Table II) is due to the formation of reducing sugar, as explained earlier.

Since the direct interconversion of related disaccharides is obviously a reversible process, an experiment was devised in which sucrose would be produced rather than consumed. This was particularly important, since the evidence for the identity of the non-reducing disaccharide produced from sucrose and sorbose was entirely circumstantial and not based on the isolation and identification of the sugar. Sucrose, on the other hand, can be identified with a fair degree of certainty with invertase.

A phosphate-free enzyme preparation was allowed to act on a mixture containing 2 per cent fructose and 2 per cent α-D-glucosido-β-D-ketoxyloside which had been previously synthesized from glucose-1-phosphate and

ketoxylose (5). Sucrose was estimated with invertase and glucosido-ketoxyloside by acid hydrolysis in the same manner as was glucosido-sorboside in the previous experiments.

After 1 hour of incubation at 30°, 2.5 (± 0.3) mg. of sucrose were formed per ml. and a total of 4.8 (± 0.3) mg. per ml. was found after 3 hours. Approximately equivalent amounts of glucosidoketoxyloside disappeared.

The initially observed rate of sucrose synthesis was found to be less than half of the maximum rate observed when glucose-1-phosphate was used as substrate in place of glucosidoketoxyloside in the presence of fructose. Since the reversible reaction between glucose-1-phosphate and ketoxylose is known to be slow (3), it may be inferred that the rate of decomposition of glucosidoketoxyloside rather than the rate of sucrose synthesis limited the total rate of interconversion of the disaccharides.

DISCUSSION

There can be no doubt that both the phenomena of isotope exchange between inorganic and organic phosphate and the interconversion of disaccharides in the absence of phosphate are due to one and the same enzyme, which has been called "sucrose phosphorylase," if the following considerations are taken into account: (1) Both processes are carried out vigorously by sucrose phosphorylase preparations which are virtually devoid of most other enzymes. (2) Both reactions are inhibited by glucose. (3) Most significantly, the presence of phosphate depresses the rate of interconversion of disaccharides, indicating that phosphate competes with the ketoses for the enzyme which catalyzes this process.

It appears, then, that the rôle of the enzyme is to combine reversibly with the glucose residue of glucose-1-phosphate or of those disaccharides which can act as substrates, and to release the esterified phosphate or glycosidically bound sugar. The enzyme-glucose complex must retain the energy of the phosphate or glycosidic bond. The sucrose phosphorylase may, therefore, be considered as a glucose-transferring system which can react with a rather remarkable variety of substrates. In addition to reacting with phosphate and carbonyl groups of certain ketoses, the enzyme has been found to catalyze the addition of glucose to the secondary alcoholic group of at least one aldose (7).

The reversible phosphorolysis of sucrose can now be interpreted as consisting of the following set of reactions.

$$\text{Glucose-1-fructoside} + \text{enzyme} \underset{}{\overset{\pm \text{ fructose}}{\rightleftharpoons}} \text{glucose-enzyme}$$
$$\text{(sucrose)}$$
$$\Updownarrow \pm \text{ phosphate}$$
$$\text{glucose-1-phosphate} + \text{enzyme}$$
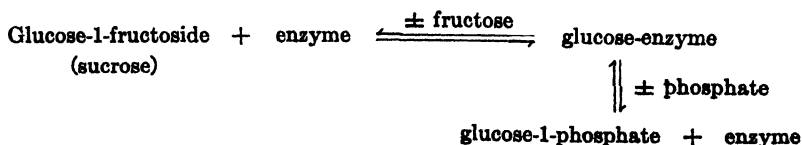
The direct interconversion of disaccharides may be illustrated by the production of sucrose from glucosidoketoxyloside:

$$\text{Glucose-1-ketoxyloside} \;+\; \text{enzyme} \xrightleftharpoons[]{\pm \overset{s}{\text{ketoxylose}}} \text{glucose-enzyme}$$

$$\Updownarrow \pm \text{fructose}$$

$$\text{glucose-1-fructoside} \;+\; \text{enzyme}$$
$$\text{(sucrose)}$$
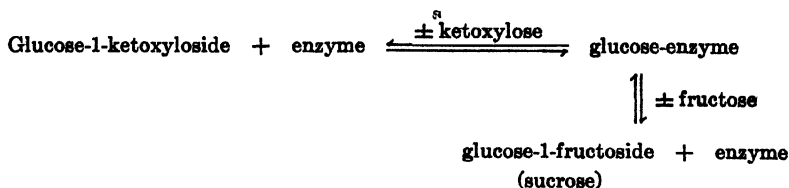
The demonstration that the accumulation of glucose-1-phosphate is not necessary for the conversion of disaccharides does not preclude the possibility that phosphate does enter into the enzymatic reaction, possibly as a firmly bound coenzyme. It is impossible to tell whether the protein itself transfers the "energy-rich glucose" or whether a coenzyme which is closely associated with the protein is involved. It is possible, for instance, to visualize a carbohydrate residue which would be directly concerned with the glucose transfer and would act as a glucose-carrying coenzyme.

The direct exchange of glycosidic linkages has been observed with other bacterial enzymes and discussed in previous papers (8, 9). Thus, neither the production of dextran nor of levan from sucrose requires the accumulation of phosphoric esters. The enzymes which catalyze these reactions appear simply to exchange a glucosidofructoside linkage for a 1:6-glucosidoglucose bond in one case or for a 2:6-fructosidofructose bond ·in the other.

Sucrose phosphorylase seems to belong to the same class of enzymes, although it has the additional power to attack the phosphate bond. It is quite possible that the better known phosphorylases which are involved in the synthesis of starch and glycogen are essentially similar in their mode of action. Since the transfer of glucose may be compared to the well known transmethylation and transamination reactions, as well as to the transfer of hydrogen atoms in biological oxidations, it would seem appropriate to consider sucrose phosporylase as a "transglucosidase." The general type of enzyme involved in the exchange of glycosidic bonds might then be called "transglycosidase."

It is quite possible that the production of many disaccharides and polysaccharides in plant and animal tissues may depend on transfers of "energy-rich" sugar residues without the intermediate accumulation of phosphoric esters (9). The remarkable versatility of the sucrose phosphorylase suggests that one and the same enzyme might, in some cases, account for the formation of a number of different compounds.

The direct synthesis of sucrose from glucosidoketoxyloside adds a third mechanism of biological synthesis of sucrose to those already studied in our

laboratory (1, 8).   It is, in reality, but a variant of the mechanism postulated from the indirect evidence of the reversible nature of levan synthesis.

The strong competition of glucose with both glucose-1-phosphate and sucrose would indicate that the carbonyl atom of glucose is not greatly involved in the affinity of sucrose phosphorylase for the glucose portion of these compounds.   Unpublished experiments have shown that xylose, xylose-1-phosphate, arabinose, galactose, and galactose-1-phosphate have a very much weaker inhibitory action on sucrose phosphorylase than does glucose.   It seems probable, therefore, that the alcoholic groups away from the carbonyl group, possibly together with the pyranose ring structure, are important in determining the specificity of enzyme-substrate combination.

SUMMARY

1. A rapid exchange of P^{32} was found to occur between inorganic phosphate and glucose-1-phosphate in the presence of sucrose phosphorylase of *Pseudomonas saccharophila* and in the absence of ketose sugars.

2. Virtually phosphate-free preparations of sucrose phosphorylase were found capable of interconverting sucrose and its analogue, glucosido-sorboside.

3. By applying the same principle, sucrose was synthesized directly from glucosidoketoxyloside and fructose with the aid of the enzyme.

4. Sucrose phosphorylase may be considered as a versatile "transglucosidase," representing a class of enzymes which may be referred to as "transglycosidases."

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STUDIES WITH BACTERIAL SUCROSE PHOSPHORYLASE

II. ENZYMATIC SYNTHESIS OF A NEW REDUCING AND OF A NEW NON-REDUCING DISACCHARIDE*

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In addition to sucrose, which has been synthesized by the reaction between glucose-1-phosphate and fructose under the influence of "sucrose phosphorylase" from *Pseudomonas saccharophila*, two new sucrose analogues have been prepared by the use of the same enzyme (1–4). In these disaccharides, L-sorbose and D-ketoxylose, respectively, were substituted for D-fructose.

In the present studies, a survey of a number of other compounds was undertaken to determine whether they could be used as substrates for the enzyme. In the course of these studies, not only was a new analogue of sucrose, α-D-glucosido-L-ketoarabinoside, prepared, but also a new reducing disaccharide, 3-(α-D-glucosido)-L-arabinose. The remarkable dissimilarities in structure of the reducing and the non-reducing disaccharides at first made it seem unlikely that one and the same enzyme could catalyze the synthesis of both. The experimental evidence strongly indicates that sucrose phosphorylase is involved in the production of both sugars, a fact which poses interesting problems concerning the general nature of enzyme specificity.

EXPERIMENTAL

Methods

The sucrose phosphorylase preparations were obtained from dry cells of *Pseudomonas saccharophila* by methods previously described (5, 1). Glucose-1-phosphate was prepared by the phosphorolysis of starch with potato phosphorylase. Maltose-1-phosphate, D-xylose-1-phosphate, and galactose-1-phosphate were synthesized chemically (6). Of the synthetic sugars used in the experiments, L-fructose was kindly supplied by Dr. M. Wolfrom, D-mannoheptulose by Dr. C. S. Hudson, and D-ribose by Dr. J. W. Foster of Merck and Company.

To obtain crude preparations of ketopentoses, the corresponding aldose

sugars were autoclaved in the presence of phosphate buffers at or near neutrality. After passage through ion exchange columns to remove the added phosphates and acidic products of the reaction, the residual aldose was, in some cases, largely removed from the crude syrup by the addition of alcohol and crystallization at low temperature. This method had been used previously in our laboratory to prepare crude D-ketoxylose from D-xylose (4), and crystalline D-tagatose from galactose.

In the earlier studies it was thought that only the corresponding ketose was produced from its aldose analogue. However, it became apparent that, at least in the case of pentoses, a further rearrangement occurs, involving the position of the hydroxyl on the 3rd carbon atom, so that at least two ketose sugars can be derived from a single aldose. Thus, the syrup obtained after autoclaving D-arabinose was shown to contain D-ketoxylose. This became evident when sucrose phosphorylase was allowed to act on such a syrup in the presence of glucose-1-phosphate. The resulting disaccharide was identified as D-glucosido-D-ketoxyloside (see below). The formation of L-ketoarabinose from L-arabinose was proved in a similar way by the isolation of glucosido-L-ketoarabinoside after the enzymatic condensation of glucose-1-phosphate with the enolized products of L-arabinose. Identical chemical changes would be expected in the D and L isomers of the aldose when heated under identical conditions with phosphate. This expectation was supported by the observation that changes in optical rotation which occurred in heated solutions of D- and L-arabinose were quantitatively equal, but opposite in sign. From this, it may be concluded that both D-ketoxylose and D-ketoarabinose were formed from D-arabinose, even though only D-ketoxylose was demonstrated directly. Similarly, both of the L-ketopentoses are probably formed from L-arabinose, even though only L-ketoarabinose was positively identified. Ketose derivatives were prepared from D- and L-arabinose, D-lyxose, D-ribose, L-fucose, and L-rhamnose by autoclaving with phosphate.

Solutions of crude dihydroxyacetone and erythrulose were prepared from glycerol and erythrytol, respectively, by the action of *Acetobacter suboxydans*. The bacteria were grown in well aerated culture media containing yeast extract, phosphate, and the homologous substrates. They were harvested by centrifugation, washed, and resuspended in one-fourth the original volume of a medium containing 6 per cent of the substrate, small amounts of NH_4Cl, $MgSO_4$, phosphate buffer, and an excess of $CaCO_3$. These cultures were incubated with aeration until the increase in reducing value due to ketose formation had ceased. They were then freed of bacteria by centrifugation and passed through ion exchange

columns to remove inorganic salts and acidic products of bacterial metabolism.

A solution of turanose was prepared by mild acid hydrolysis of melezitose followed by passage through ion exchange columns and the removal of the liberated glucose by fermentation with washed cells of *Torula monosa*.

A mixture of short chain fructose polymers was made from dialyzed and alcohol-precipitated levan produced by *Bacillus subtilis* from sucrose (7). Three equal portions of a 10 per cent levan solution were treated with 0.1 N HCl at 37° for 1, 2, and 3 hours, respectively, mixed, and passed through ion exchange columns. The liberated fructose was removed by fermentation with washed cells of *Torula monosa*. The resulting product had a reducing value approximating 25 per cent of that obtained on complete acid hydrolysis, and presumably contained fructosans having the 2,6 linkage.

The reaction between glucose-1-phosphate and various sugars was tested by adding the two components to an enzyme preparation and measuring the liberation of inorganic phosphate (2, 5). Inorganic phosphate produced in a control, in which only glucose-1-phosphate was added to the enzyme preparation, was subtracted, to allow for phosphatase activity, which, though slight, was detectable in all preparations. The reaction between analogues of glucose-1-phosphate and fructose was tested in the same manner. When possible, the changes in reducing value of reaction mixtures were also determined to establish whether a reducing or non-reducing disaccharide was produced.

Substitutions for Glucose-1-phosphate—No enzymatic reaction whatever could be observed between D-xylose-1-phosphate or D-galactose-1-phosphate and fructose, the evolution of inorganic phosphate being identical with that in D-fructose-free controls.

With maltose-1-phosphate, the addition of D-fructose to the reaction mixture caused a slightly greater production of phosphate than that observed in the control. This reaction, however, was so slow that it was impossible to establish whether maltose-1-phosphate itself was involved in a direct condensation with fructose. This was especially true, since glucose-1-phosphate may have been present as an impurity in the maltose phosphate or have been produced by the slow hydrolysis of the maltose linkage by the enzyme preparation.

That melibiose-1-phosphate cannot react with fructose was shown by the inability of the enzyme to phosphorolyze raffinose.

Formation of D-*Glucosido-*D-*ketoxyloside*—The preparation and properties of crystalline α-D-glucosido-β-D-ketoxyloside has already been described in detail (4). The crude syrup containing D-ketoxylose, which had been used

for the first synthesis, had been made by autoclaving D-xylose in the presence of phosphate and partially removing the parent sugar by crystallization.[1]

No reaction whatever could be observed when D-arabinose was added to glucose-1-phosphate and enzyme. When, however, solutions of D-arabinose were enolized with phosphate, the resulting mixture of sugars caused a significant production of inorganic phosphate from glucose-1-phosphate, accompanied by a decrease in the total reducing value. To identify the disaccharide produced in this reaction, the experiment was carried out on a large scale and the product isolated in crystalline form. 15.5 gm. of syrup were prepared by autoclaving D-arabinose with phosphate, passing the solution through ion exchange columns, concentrating it *in vacuo*, and removing a large portion of unconverted D-arabinose by crystallization in the cold. This syrup was allowed to react with 33 gm. of glucose-1-phosphate and an enzyme preparation from 3 gm. of dry cells of *Pseudomonas saccharophila*. The mixture was incubated for 12 hours at 37° and pH 7.5 in the presence of 0.1 M barium acetate. The reaction mixture was then treated in the manner previously described (4), the reducing sugars being removed by extraction of their osazone derivatives with ethyl acetate. A yield of 0.435 gm. of crystalline non-reducing disaccharide was obtained in this reaction. This disaccharide was proved to be identical with the previously described α-D-glucosido-β-D-ketoxyloside.[2]

The identification of the synthetic sugar as a ketoxyloside proves that D-ketoxylose is formed from D-arabinose upon treatment with phosphate at high temperature. All attempts to demonstrate the formation of a ketoarabinoside were unsuccessful. Since D-ketoarabinose was probably present in the reaction mixture (see the discussion above), the negative evidence indicates that this sugar cannot function as a glucose acceptor with this enzyme.[3]

[1] It would be expected that D-lyxose, like D-xylose, would yield D-ketoxylose after similar treatment   A solution of D-lyxose was autoclaved with phosphate buffer at pH 7 0, passed through ion exchange columns, and added to a mixture of phosphorylase and glucose-1-phosphate   There resulted a rapid liberation of phosphate which was comparable to that described in experiments with the syrup obtained from D-xylose   D-Lyxose, itself, like D-xylose, was inactive

[2] Identical specific rotation before and after hydrolysis; identical x-ray diffraction pattern, ketoxylose component identified as the osazone upon hydrolysis of the disaccharide and removal of glucose by fermentation.

[3] The close relationship between D-arabinose and D-ribose would suggest that identical ketose derivatives would be obtained from the two sugars on heating with phosphate. When D-ribose was thus treated and the resulting mixture used as substrate for the enzyme, a rapid evolution of phosphate from glucose-1-phosphate was observed, presumably owing to the formation of glucosidoketoxyloside. The addition of untreated D-ribose, unlike that of D-arabinose, resulted in a very slight

Enzymatic Synthesis of Reducing Disaccharide, 3-(α-D-Glucosido)-L-arabinose—Since all of the previously studied condensation reactions catalyzed by sucrose phosphorylase involve the reaction of glucose-1-phosphate or a suitable disaccharide with a ketose, it was surprising to find that the addition of L-arabinose, an aldose sugar, to enzyme preparations resulted in a relatively slow but continued evolution of phosphate from glucose-1-phosphate. Instead of a decrease in reducing value, such as had been observed in the formation of sucrose analogues, an increase in reducing value was observed in the reaction with L-arabinose.

The estimation of glucose in the reaction mixtures by fermentation with *Torula monosa* showed that the observed increase in reducing value was not accounted for by any direct or indirect production of glucose from glucose-1-phosphate, nor was there any evidence of easily hydrolyzable non-reducing disaccharide among the products of the reaction.

To determine what compound was formed in the condensation, a mixture of L-arabinose and glucose-1-phosphate was incubated in the presence of the enzyme.[4] The unused glucose-1-phosphate was removed by precipitation with alcohol, and the unused L-arabinose by fermentation with a selected strain of *Escherichia coli*. The removal by extraction of its osazone derivative was precluded by the reducing nature of the synthesized product. After passage through ion exchange columns and concentration of the solution *in vacuo*, the remaining sugar was crystallized from alcohol. This was found to be a reducing disaccharide, D-glucosido-L-arabinose. Unlike the sucrose analogues previously prepared with the aid of the enzyme, the new sugar is not easily hydrolyzed with acid and does not give the "Raybin"
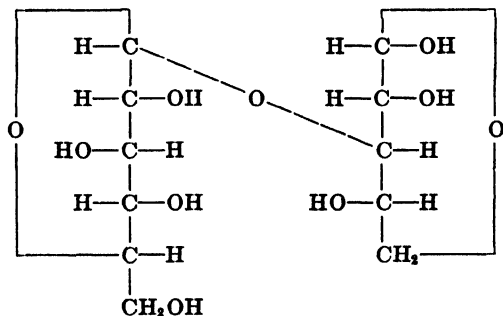
(I)

production of phosphate. This reaction was too weak, however, to determine whether the ribose itself was involved, since the quantity of the sugar available was small and its purity was not established.

[4] A complete description of the method of preparation, study of the structure, and properties of the new disaccharide will be published elsewhere.

reaction with diazouracil characteristic of the sucrose linkage.   A study of its derivatives indicates that its structure is 3-(α-D-glucosido)-L-arabinose with both the hexose and the pentose units in the pyranose (six-membered ring) form.   The formula may be written as in (I).

It seemed exceptional for a single enzyme to catalyze two such diverse reactions as that involving the 2nd (carbonyl) carbon of a ketose on the one hand, and the 3rd (alcohol) carbon of an aldose, on the other.   It was, therefore, necessary to eliminate the possibility that the enyzme preparations might contain two different phosphorolytic enzymes which carry out the two reactions.

That sucrose phosphorylase is involved in the reaction with L-arabinose was established beyond reasonable doubt by the following experiment.

TABLE I

Initial Rate of Inorganic Phosphate Production from Glucose-1-phosphate in Presence of Sucrose Phosphorylase upon Addition of Fructose and Arabinose

Dilute enzyme preparation incubated with 0 1 M glucose-1-phosphate with the additions indicated below at 30°.

Additions	Rate of total evolution of phosphate, micromoles per ml per hr.
M/4 L-arabinose	2.1 (±0.2)
M/64 D-fructose	3.1 (±0.2)
M/64 " + M/4 L-arabinose	4.1 (±0.2)
M/4 " . .	9.7 (±0.2)
M/4 " + M/4 L-arabinose	8 6 (±0.2)

D-Fructose and L-arabinose were added separately and in combination to samples of a solution containing glucose-1-phosphate and enzyme. The D-fructose concentrations were so selected as to give the maximum rate of sucrose formation, in one case, and a lower rate, in which the D-fructose concentration was the limiting factor, in the other.   The total initial rate of phosphate evolution was measured in each case.   The results are presented in Table I.

It is apparent that, when L-arabinose is added to a mixture in which the D-fructose concentration is insufficient to allow the maximum rate of reaction, an increase in the total utilization of glucose-1-phosphate results. The same amount of L-arabinose, however, when added to a mixture in which the D-fructose concentration is not limiting, causes a decrease in the total rate of phosphate evolution.   This indicates clearly that both D-fructose and L-arabinose are competing for the same enyzme.

Additional evidence that the same enzyme is involved in reactions with both D-fructose and L-arabinose may be summarized as follows: (1) The

enzyme catalyzing both reactions is produced to a marked extent when sucrose is used as substrate for the growth of the organisms, but not when D-glucose or L-arabinose is used. (2) The relative rates of reaction with D-fructose and L-arabinose, respectively, remain constant after partial inactivation by heat (see Table II). (3) In the preparation and partial purification of phosphorylase from the crude extract of dried cells, the relative rates of reaction with D-fructose and L-arabinose remain constant, although the total activity towards both is considerably decreased and many other enzymes are destroyed or removed. (4) On fractionation of the enzyme preparation with ammonium sulfate (33 to 40 per cent saturation; 40 to 45 per cent saturation; 45 to 63 per cent saturation), the relative activities of the three fractions are the same for both sugars.

TABLE II

Effect of Various Treatments of Sucrose Phosphorylase Preparations on Initial Rate of Inorganic Phosphate Formation from Glucose-1-phosphate in Presence of Fructose and of L-Arabinose, Respectively

	With fructose	With arabinose
1 Initial activity remaining after heating at 55–56° for 6 min , %	63 (± 2)	65 (± 3)
2 Initial activity of dry cell extract remaining after partial purification of preparation by repeated precipitations, %	27 (± 2)	26 (± 2)
3 Total recovered activity of preparation appearing in fractions precipitated with $(NH_4)_2SO_4$, %		
(a) 0 33–0 40 saturation . . .	36 (± 2)	38 (± 2)
(b) 0.40–0.45 " .	44 (± 2)	42 (± 2)
(c) 0.45–0.63 "	20 (± 2)	20 (± 2)

The above considerations indicate not only that the same enzyme is involved in both reactions, but also that no additional enzyme is required for the formation of D-glucosido-L-arabinose. Technical difficulties have made it impossible so far to determine the equilibrium constant for the reaction, D-glucose-1-phosphate + L-arabinose ⇌ D-glucosido-L-arabinose + inorganic phosphate. The principal difficulty is that the reaction becomes exceedingly slow as the L-arabinose concentration decreases or when considerable inorganic phosphate is produced from glucose-1-phosphate. From a few experiments it is apparent that the equilibrium may be very much further to the right than that for sucrose synthesis. The reaction from right to left is extremely slow; it is necessary to use relatively large amounts of synthetic disaccharide and inorganic phosphate to detect the reaction at all.

Synthesis of α-D-*Glucosido-β-L-ketoarabinoside*—When enolized L-arabi-nose was tested as substrate for sucrose phosphorylase activity, it became apparent that a reaction diffcrent from that observed with L-arabinose takes place.

The L-arabinose was autoclaved in the presence of phosphate and the resulting solution was treated according to the methods already described to remove inorganic salts and as much of the parent sugar as possible.

When the resulting syrup was added to the enzyme preparation together with glucose-1-phosphate, an evolution of inorganic phosphate was ob-served. Although the initial rate of reaction appeared to be approximately equal to that observed with L-arabinose, the phosphate production soon stopped, indicating that an equilibrium condition had been reached, as in the case of D-fructose, L-sorbose, and L-ketoxylose (2, 5).[5] Instead of an increase in reducing value such as had been observed with L-arabinose, a decrease was noted in the reaction with the cnolized syrup. To obtain the disaccharide in pure form, the same general procedure was used as that described for the isolation of glucosido-D-ketoxyloside, except that L-arabi-nose was used in place of D-xylose and D-arabinose. About 800 mg. of non-reducing, easily hydrolyzable crystalline disaccharide were thus obtained. On hydrolysis, the sugar was shown to yield D-glucose and L-ketoarabinose, which was identified through its osazone derivative. This disaccharide, like the other sucrose analogues prepared with the phos-phorylase, gave the Raybin reaction. The disaccharide may be assumed to be α-D-glucosido-β-L-ketoarabinoside and to have the structure shown in (II). The purity of the isolated compound indicates that no D-gluco-

$$
\begin{array}{c}
\text{CH}_2\text{OH} \\
| \\
\text{H—C———O———C} \\
| \qquad\qquad\quad | \\
\text{H—C—OH} \quad \text{HO—C—H} \\
| \qquad\qquad\quad | \\
\text{O} \quad \text{HO—C—H} \quad \text{HO—C—H} \quad \text{O} \\
| \qquad\qquad\quad | \\
\text{H—C—OH} \quad \text{CH}_2 \\
| \\
\text{C—H} \\
| \\
\text{CH}_2\text{OH}
\end{array}
$$

(II)

sido-L-ketoxyloside is formed in the reaction. Since ketoxylose may be assumed to be present in the enolized syrup on the basis of the earlier

_____

[5] Unfortunately, it has been impossible to determine the equilibrium constant for the reaction, since the relative amounts of the various sugars in the crude syrup cannot yet be determined.

experiment, it seems likely that the L isomer of this compound is not a suitable substrate for the enzyme.

Reaction with Other Carbohydrates—In experiments outlined above and reported previously (5), it has been shown that the following sugars and their derivatives do not show any reaction with glucose-1-phosphate in the presence of sucrose phosphorylase: D-glucose, D-mannose, D-galactose, fructose-6-phosphate, fructose-1,6-diphosphate, D-arabinose, D-xylose, and D-lyxose. The reaction with D-ribose is doubtful and at best very weak. In addition, there is strong circumstantial evidence that neither D-keto-arabinose nor L-ketoxylose is a suitable substrate for the enzyme. The following carbohydrates were also tried and showed no increase in phosphate liberation from glucose-1-phosphate: D-tagatose, L-fructose, D-mannoheptulose, L-fucose, L-rhamnose, degraded levan, and turanose. The non-reactivity of turanose was also shown by the inability of the enzyme to phosphorolyze melezitose.

When L-rhamnose and L-fucose were autoclaved in phosphate buffer and added to mixtures of phosphorylase and glucose-1-phosphate, a slight reaction was observed with the former and a rather pronounced production of inorganic phosphate with the latter. It seems most likely that methyl-ketopentoses were produced by enolization of the methyl aldopentoses and that such a compound derived from fucose was readily utilized as substrate. The experience with D- and L-arabinose shows that, at least in some cases, more than one ketose may be formed by such treatment of an aldose. While it may, therefore, be postulated that the product or products of condensation of enolized methyl pentoses are glucosidomethylketopentoside analogues of sucrose, their true composition is not yet known and can only be established by their isolation in pure form.

When crude dihydroxyacetone and erythrulose preparations were used as substrates, a very slow evolution of inorganic phosphate from glucose-1-phosphate was observed. It could not be established, however, that this was due to phosphorylase activity, since the compounds are very active chemically and appeared to act on glucose-1-phosphate even in the absence of enzyme. On prolonged incubation, and particularly on heating, mixtures of these sugars with glucose-1-phosphate become acid and show the presence of inorganic phosphate. The slight increase in phosphate production with the enzyme may well be due either to the direct interaction of the ketoses with glucose-1-phosphate or to the indirect effect of pH changes on the phosphatase activity of the preparations.

DISCUSSION

It has been shown that sucrose phosphorylase may be regarded as a "transglucosidase," which catalyzes the transfer of "glucose" to a variety of acceptors (8). The enzyme appears to be very specific with regard to

the glucose portion of its substrates. The "glucose acceptors," however, show a considerable diversity of structure. So far, three types can be

TABLE III

Reactions with Glucose-1-phosphate in Presence of Sucrose Phosphorylase

Class	Compounds reacting (inorganic phosphate evolved from glucose-1-phosphate)	Compounds showing no reaction or doubtful reaction*
Ketotriose		Dihydroxyacetone†
Ketotetrose		Erythrulose†
Aldopentoses		D-Xylose
		D-Lyxose
		D-Ribose‡
	L-Arabinose	D-Arabinose
Ketopentoses	D-Ketoxylose	L-Ketoxylose?§
	L-Ketoarabinose	D-Ketoarabinose?§
Methyl pentoses	Undetermined methyl ketopentose‖	L-Fucose
		L-Rhamnose
Aldohexoses		D-Glucose
		D-Mannose
		D-Galactose
Ketohexoses	D-Fructose	L-Fructose
	L-Sorbose	
		D-Tagatose
		D-Mannoheptulose
Fructose derivatives		Fructose-6-phosphate
		Fructose-1,6-diphosphate
		Turanose (3-(glucosido)-D-fructose)
		Degraded levans (6-(fructosido)-D-fructose and polymers)

* No reaction recorded when phosphate evolution from glucose-1-phosphate in presence of M/6 substrate falls within the experimental error of the determination and is less than 1 per cent of the rate with fructose.

† Very slight evolution of phosphate observed but cannot be directly attributed to phosphorylase.

‡ Apparent slight reaction, but purity of D-ribose not certain.

§ Circumstantial evidence only that these sugars do not react based on isolation of pure glucosido-D-ketoxyloside from phosphate-treated D-arabinose and of glucosido-L-ketoarabinoside from phosphate-treated L-arabinose.

‖ Phosphate-treated L-fucose.

distinguished: (1) inorganic phosphate; (2) ketose sugars; (3) an aldose sugar, L-arabinose.

Phosphate, which appears to be the best acceptor for the "energy-rich glucose," is quite different from both of the other types in size and shape.

The ability of the enzyme to use arsenate in place of phosphate has not yet been tested.

It seems appropriate at this point to compare the various sugars which can be substituted for the phosphate group of glucose-1-phosphate to see what features they may have in common (Table III).

The ketoses which can act as glucose acceptors have a general structural similarity, but there are certain exceptions which make it impossible to define exactly the structural specificity which makes them suitable substrates for the enzyme.

It is obvious that large radicals attached to the ketoses may make them unsuitable as substrates. Thus, the phosphate radicals of the fructose phosphates, as well as the sugars glycosidically bound to fructose in turanose and degraded levulan, prevent the fructose from reacting, even though in some of these compounds the fructose is known to exist in the furanose form.

A general formula for the first three disaccharides synthesized with .the aid of the enzyme may be written as indicated (III). In sucrose, R is a

(III)
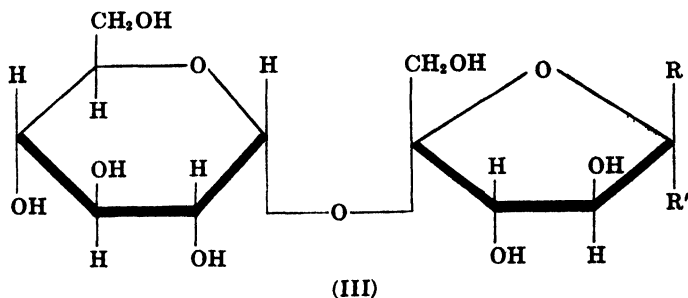
hydrogen atom, while R' is a primary alcohol group. In D-glucosido-α-L-sorboside, R is a primary alcohol group, while R' is a hydrogen atom. In glucosido-β-D-ketoxyloside, both R and R' are hydrogen atoms.

The formula of the new non-reducing disaccharide, glucosido-β-L-keto-arabinoside may be represented as in (IV). It will be seen that this differs

(IV)
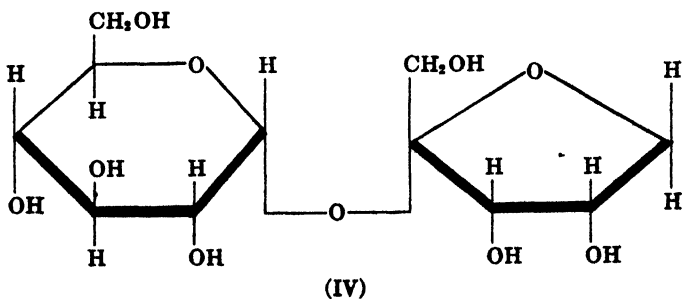
from the formula for glucosido-D-ketoxyloside only in the configuration of hydrogen and hydroxyl groups about the 4th carbon atom of the pentose. The common features of the various ketoses which have been found to react may be, therefore, diagrammatically represented as in (V). In

(V)
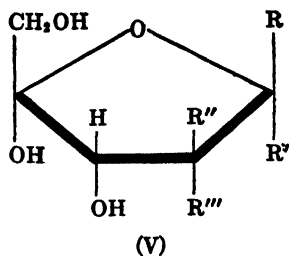
this diagram R and R′ represent either hydrogen or primary alcohol groups, while R″ and R‴ represent hydrogen or hydroxyl groups.

The above structure is not present in L-fructose, L-ketoxylose, or D-ketoarabinose, which sugars are apparently unable to act as substrates for the enzyme. On the other hand, a similar structure does exist in the furanose (five-membered ring) form of D-tagatose and of D-mannoheptulose. However, neither of these sugars reacts in the presence of the enzyme. This is somewhat surprising, since the non-reacting D-tagatose bears the same relationship to the reacting L-ketoarabinose as D-fructose does to D-ketoxylose. It appears that with these compounds the relative positions of the terminal primary alcohol group and the adjacent hydroxyl groups are important, no reaction taking place if they are at R′ and R‴ respectively. The additional alcoholic group in mannoheptulose may well interfere with the reaction. Besides, it is not known whether either sugar exists to any extent in the furanose form which is stabilized in the non-reducing disaccharides and might well be a prerequisite for the reaction to take place.

The structure of 3-(α-D-glucosido)-L-arabinose is so fundamentally different from that of the reducing sugars that no obvious conclusions can be drawn as to the chemical configurations responsible for the affinity of the enzyme for L-arabinose. Not only is a secondary alcoholic group instead of a carbonyl group involved in the linkage, but the sugar is in the pyranose rather than the furanose form. For comparison with the non-reducing disaccharides, its structural formula is shown in (VI). It is unlikely that the phosphorylase causes a primary condensation of D-glucose with L-arabinose through the carbonyl atoms of both and that a rearrangement takes place later. A spontaneous rearrangement of this sort would not be expected and, indeed, an analogue of the possible primary condensation product, D-xylosido-D-glucoside, is known to exist as a stable compound

(VI)
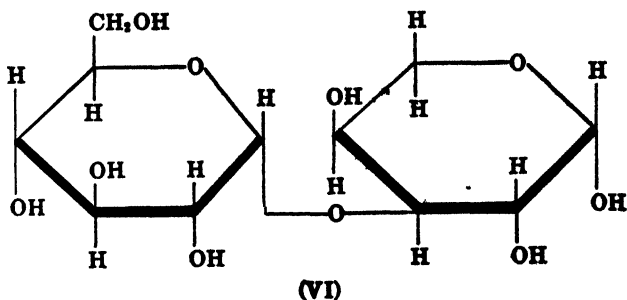
(9). That an enzymatic rearrangement does not take place is indicated by evidence which has been presented that a single enzyme is involved in the synthesis of glucosido-L-arabinose. .

The demonstration that one and the same enzyme may catalyze such apparently dissimilar reactions as the syntheses of reducing and non-reducing disaccharides and of glucose-1-phosphate indicates that super-ficially dissimilar compounds or linkages may depend on a single enzyme for their production. It also indicates that at least in some cases the affinity of an enzyme for a substrate does not depend on the obvious chemical groups of the latter but possibly on subtle spacial relations which can only be elucidated by further study.

The available evidence indicates that the equilibrium in the reaction, D-glucose-1-phosphate + L-arabinose ⇌ D-glucosido-L-arabinose + phosphate, is far to the right as compared with the equilibrium shown for sucrose synthesis.[6] The difference in the apparent equilibrium could be explained if the bond energy of the reducing disaccharide were smaller than that of sucrose. No definite information regarding this point could be found. However, with phosphate esters (glucose-1-phosphate, glucose-6-phosphate) it is known that the linkage to the carbonyl group has a higher energy content than that to an alcoholic group. Similar relations probably apply to glycosidic linkages of reducing and non-reducing disaccharides. This has been postulated as one of the reasons for the almost irreversible nature of the reactions involved in the synthesis of dextran and levan from sucrose (8). Another consideration which must be taken into account in explaining the difference in apparent equilibria is the possibility that only

[6] In a previous estimation of the equilibrium constant for the reaction between glucose-1-phosphate and D-ketoxylose (2), the concentration of D-ketoxylose in the experimental solutions was determined by polarimetric analysis on the assumption that only one ketose is formed to any extent by enolization of a given aldose with phosphate. In view of the demonstration that more than one ketose may be formed on autoclaving arabinose in the presence of phosphate, the previously determined equilibrium constant for this reaction may be inaccurate.

the furanose form of sugars like D-fructose and L-sorbose is involved in the reaction and this form constitutes only a fraction of the total amount of ketose present at equilibrium.

The synthesis of D-glucosido-L-arabinose appears to be the first example of an *in vitro* enzymatic synthesis of a reducing disaccharide directly from hexose units. The only other reducing disaccharide which has been synthesized enzymatically *in vitro* is maltose, formed from glucose-1-phosphate by the successive use of starch phosphorylase and amylase.

SUMMARY

. 1. A new non-reducing disaccharide has been prepared from glucose-1-phosphate and enolized L-arabinose with the aid of sucrose phosphorylase preparations from *Pseudomonas saccharophila*. This disaccharide has been identified as α-D-glucosido-β-L-ketoarabinoside. It is analogous to sucrose in structure and certain chemical reactions.

2. A new reducing disaccharide has been synthesized from glucose-1-phosphate and L-arabinose with the same enzyme preparations. This has been identified as 3-(α-D-glucosido)-L-arabinose. Unlike sucrose and its analogues, this sugar has a free carbonyl group, is difficultly hydrolyzable with acid, does not give the Raybin reaction with diazouracil, and contains both monosaccharide components in the pyranose (six-membered ring) form.

3. It has been demonstrated that both the reducing and non-reducing disaccharides are produced by the same enzyme and the possibility that an additional enzyme may be involved in the synthesis of D-glucosido-L-arabinose has been shown to be remote.

4. A number of other compounds have been tested as substrates for sucrose phosphorylase, both as substitutes for D-glucose-1-phosphate and for D-fructose.

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AN ENZYMATIC METHOD FOR THE DETERMINATION OF URIC ACID IN WHOLE BLOOD*

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In 1939 Blauch and Koch (1) published an enzymatic method for the colorimetric determination of uric acid in whole blood. This method involves the use of the enzyme uricase for the specific destruction of uric acid. "True uric acid" values were obtained by the difference in the intensity of color produced before and after incubation with the enzyme. The authors reported "true uric acid" values for normal whole blood averaging 2 mg. per cent and non-uric acid chromogenic values, calculated as uric acid, 1 mg. per cent. Recoveries of uric acid added to whole blood before deproteinization averaged 93.5 per cent.

Bulger and Johns (2) later published a method similar in principle to that of Blauch and Koch. "True uric acid" was estimated by determining the reduction of alkaline ferricyanide by protein-free filtrates of plasma before and after uricase action. Recoveries of uric acid added to plasma were quantitative. Average values for normal plasma were 4 mg. per cent of "true uric acid" and 1.7 mg. per cent of non-uric acid. These workers suggested, as an explanation of the discrepancy between their results and those obtained by the method of Blauch and Koch (1), that in the latter procedure there is a depression of color development with phosphotungstic acid due to the presence of substances in the tungstate filtrate of blood, which do not, however, influence the reduction of alkaline ferricyanide. A colorimetric modification of the Bulger and Johns method has been recently published by Silverman and Gubernick (3). Lower non-uric acid values than Bulger and Johns reported were obtained by these workers (less than 1.0 mg. per cent); however, this procedure is still not applicable to whole blood.

In view of the fact that there are many inherent errors in the Blauch and Koch (1) method and that the Bulger and Johns (2, 3) procedure is somewhat involved and cannot be applied to whole blood, we thought it worth while to reinvestigate the problem and to attempt to develop a specific

method for the determination of uric acid which could be applied to whole blood, as well as to plasma and serum, and to adapt the method to the photoelectric colorimeter.

Early in the investigation it was found that if the Folin reagents (4), namely phosphotungstic acid and urea-cyanide, were used, a constant center setting on the photoelectric colorimeter could not be obtained because of a gradual development of color due to the reagents. Blauch and Koch (1) experienced the same difficulty. However, it was found by one of us in previous work (5) that by employing the arsenophosphotung-stic acid reagent of Benedict (6) and the urea-cyanide reagent of Christman and Ravwitch (7) no color developed due to the reagents and a constant center setting could be obtained on the colorimeter.

Accordingly, these reagents were employed and a standard uric acid calibration curve was established. The desired volume of standard uric acid solution (lithium carbonate standard) (5) was measured into a 25 cc. volumetric flask, 2.5 cc. of urea cyanide and 2.0 cc. of arsenophosphotung-stic acid (6) (diluted 1:4) were added, and the flasks were immediately made to volume. The color was read at 40 minutes in the photoelectric colorimeter with Filter 660. It was found that the color did not follow Beer's law over the desired range of concentrations, so that it was necessary to use a calibration curve.

Using the curve thus obtained, we applied this direct procedure to whole blood and to plasma. It was found that if varying amounts of the same filtrate of whole blood (3 to 10 cc.) were analyzed, different values in terms of mg. per 100 cc. were obtained. However, with plasma filtrates the uric acid values did not show as great a variation (Table I). In addition, for a fixed amount of filtrate recoveries of added uric acid varied from 95 to 130 per cent.

In view of these findings it was apparent that this direct procedure with uricase could not be used, first because the recoveries of uric acid added to whole blood and plasma were inaccurate and, second, because the directly determined uric acid in whole blood varied with the volume of protein-free filtrate employed (Table I). This factor is particularly important in view of the fact that 5 cc. of protein-free filtrate are used for the determination of total chromogenic value and 10 cc. for the non-uric acid chromogenic value.

Discrepancies in uric acid values found in whole blood and serum have been previously reported (8, 9), and it has been felt that the method for whole blood is less reliable because of the concentration of interfering substance in the red cells. Ergothioneine and glutathione both exist in high concentrations in the human red cell. Because these substances are removed in the Newton (10) method for the determination of uric acid in

blood, by a preliminary precipitation of ergothioneine and glutathione with lithium chloride and silver nitrate, an investigation of the method was carried out. However, in this procedure arsenotungstic acid and urea cyanide are employed for the development of color. Since we were unable to obtain the same intensity for constant concentrations of uric acid with different preparations of arsenotungstic acid, arsenophosphotungstic acid (6) and the urea-cyanide, devised by Christman and Ravwitch (7), were substituted. The removal of ergothioneine and glutathione with silver as suggested by Newton (10) was adopted. The silver precipitation was modified so as to allow accurate pipetting of samples instead of decanting; that is, twice the amount of filtrate desired was treated with

TABLE I

Uric Acid Concentration in Varying Amounts of Filtrate

| | Volume of filtrate | Uric acid | |
		Direct method	Modified Newton method
	cc.	*mg. per cent*	*mg. per cent*
Whole blood	3	4.61	4.72
	5	4.79	4.89
	10	5.38	4.75
Plasma	3	6.11	6.78
	5	6.21	6.87
	10	6.42	6.84
Serum	3		5.75
	5		5.78
	10		5.69

lithium chloride and silver nitrate solutions and one-half the total volume of supernatant fluid taken for analysis.

A calibration curve was established with a lithium carbonate-uric acid standard (5). To each aliquot of this standard solution which contained a different amount of uric acid, 1 cc. of hydrochloric acid (3.5 per cent by volume) was added. The addition of this amount of hydrochloric acid approximated the acid concentration of a blood filtrate after the removal of ergothioneine and glutathione with lithium chloride and silver nitrate. This was necessary, since it was found that varying amounts of acid influenced the color development. With this modification the following procedure was employed: 2 cc. of arsenophosphotungstic acid diluted 1:4, 2.5 cc. of the urea-cyanide mixture, and 1 cc. of a 3.5 per cent solution of HCl were added to an aliquot of a standard uric acid solution in a 25 cc. vol-

umetric flask, which was immediately made to volume. The color was read in the Evelyn photoelectric colorimeter with Filter 660 after 40 minutes, at which time the color was constant and maximum. A constant center setting was obtained with a constant K value (0.0134) for concentrations of uric acid ranging from 10 to 60 γ (Table II).

The modified Newton procedure was applied to whole blood, plasma, and serum. It was found that different amounts (3 to 10 cc.) of tungstic acid filtrate gave similar values for uric acid when expressed as mg. per cent (Table I). These findings suggest that glutathione and ergothioneine may account for the disproportionality observed when different

TABLE II

Reproducibility of Chemical Method for Determination of Lithium Urate and Constancy of Ratio of Photometric Density to Concentration of Compound

Lithium urate present	No. of tests	Range of galvanometer reading*	Average photometric density (L value)†	Calculated K value‡
γ				
10	4	73², 74 .	0.1323	0.0132
20	5	53°, 54³	0.2686	0.0134
30	5	38°, 40	0 4090	0.0136
40	5	29°, 30¹	0.5290	0.0132
50	5	22, 22³	0.6505	0.0130
60	4	16, 16³	0 7860	0.0131
Average				0 0134

* These values correspond to the maximal blue color produced when taken with a center setting of 79³ and Filter 660.

† This is analogous to optical density as measured on a spectrophotometer and corresponds to the quantity (2 − log 10 of the galvanometer reading).

‡ These values are obtained by dividing the photometric density by the amount of the lithium urate in the test solution.

amounts of filtrate in which they are present are analyzed by the direct method (Table I). To investigate this possibility, known amounts of glutathione and ergothioneine were added to pure uric acid solutions of known concentration and their effect on the color development determined. The direct method of Benedict, modified as previously described, with 2 cc. of arsenophosphotungstic acid and 2.5 cc. of urea-cyanide, was used (Table III).

Although the present study in some cases deals with amounts of these substances larger than those normally present in blood, the results show that, while glutathione itself gives no measurable uric acid color, the addition of this substance to a pure solution of uric acid or to a pure

solution of ergothioneine, or to a solution of uric acid plus ergothioneine does cause a definite increased production of color. The increase in color produced becomes greater as larger amounts of glutathione are added.

TABLE III

Effect of Ergothioneine and Glutathione on Color Value of Uric Acid in Pure Solutions

Substance			Color value expressed as uric acid			
Uric acid	Ergothioneine	Glutathione	Found	Theoretical*	Increased production	Depression
γ	γ	γ	γ	γ	*per cent*	*per cent*
0		30	0	0		
0		50	0	0		
0		100	0	0		
0		150	0	0		
0	30		8.65			
0	50		14.52			
0	100		29.46			
20	30		28.68	28.65		0.0
20	50		34.21	34.52		0.9
20	100		48.29	49.46		2.3
20		30	20.00	20.00	0.0	
20		50	20.31	20.00	1.6	
20		100	23.09	20.00	15.4	
20		150	25.00	20.00	25.0	
0	30	30	10.86	8.65	25.5	
0	30	50	12.39	8.65	42.3	
0	30	100	14.11	8.65	63.1	
0	50	50	17.59	14.52	21.1	
0	50	100	22.24	14.52	53.2	
0	50	150	23.09	14.52	59.0	
0	100	100	39.01	29.46	32.5	
0	100	150	43.55	29.46	47.8	
20	30	30	29.50	28.65	2.0	
20	50	50	36.64	34.52	6.3	
20	50	100	39.84	34.52	15.4	
20	50	150	42.31	34.52	22.5	
20	100	100	56.24	49.46	13.7	
20	100	150	58.80	49.46	18.8	

* Color value of 20 γ of uric acid plus color value of increasing amounts of either ergothioneine or glutathione.

In contrast, although a pure solution of ergothioneine produces an appreciable color (approximately 30 per cent of that given by the same concentration of uric acid), when it is added to a solution of pure uric acid there is an actual depression of uric acid color, the depression being

greater with increasing amounts of ergothioneine. In solutions in which all 3 of these substances are present, the actual color produced is not the sum of the colors due to the uric acid plus ergothioneine, but that sum as modified by the depression of color intensity caused by ergothioneine and the increase in color production caused by the glutathione.

To study the effect of the addition of ergothioneine and glutathione to whole blood, the following experiment was done. Different amounts (3 to 10 cc.) of the protein-free filtrate of whole blood, whole blood plus

TABLE IV

Effect of Added Ergothioneine and Glutathione on Color Value of Uric Acid in Whole Blood

	Volume of filtrate	Color value as uric acid	Color value added	Theoretical† total color value	Increased production	Depression	Total color value expressed as uric acid
	cc	*γ*	*γ*	*γ*	*per cent*	*per cent*	*mg. per cent*
Whole blood*	3	13.84					4 61
	5	23 97					4 79
	10	53 76					5.38
Whole blood + 50 γ ergothioneine	3	16 32	4 84	18 68		12 6	5 44
	5	29.50	7 26	31 23		5.5	5.90
	10	67.41	14 52	68.28		1 3	6.74
Whole blood + 50 γ glutathione	3	13 84	0	13 84	0	0	4.61
	5	25.24	0	23 97	5 3		5 04
	10	58.80	0	53.76	9 4		5.88
Whole blood + 50 γ ergothioneine + 50 γ glutathione	3	17.45	4.84	18 68		0 6	5 82
	5	31 82	7 26	31 23	0.2		6.36
	10	71 03	14 52	68.28	4.0		7.10

* Before deproteinization (1:10 tungstic acid filtrate) 50 γ of ergothioneine (or glutathione) per cc. were added   This represents a color value of 14.52 γ expressed as uric acid (Table I) per 10 cc. of filtrate for only ergothioneine, since glutathione was found to give no color.

† Value of uric acid found in whole blood plus color value of ergothioneine added per aliquot of filtrate analyzed

50 γ of ergothioneine per cc., whole blood plus 50 γ of glutathione per cc., and whole blood plus 50 γ of both substances were analyzed by the direct method (Table IV). Interpretation of these results is complicated by the fact that both substances added are already present in blood. In spite of this, however, the results are similar to those found with pure solutions. In whole blood to which ergothioneine was added the depression of color intensity is greater with smaller volumes of filtrate, but with larger volumes the amount of glutathione originally present is larger and would be

expected to cause a greater increased production of color. In whole blood containing added glutathione the increase of color intensity becomes greater with larger amounts of filtrate analyzed. In blood to which both glutathione and ergothioneine were added, 3 cc. of the filtrate showed a slight depression of color. On the other hand, 5 cc. of filtrate showed a slight increased production of color and 10 cc. a definite increase. These results might also be expected from previous findings that with smaller amounts of glutathione the effect of ergothioneine in depressing color development may predominate but that as the amount of glutathione present increases this depression is obscured by the increased production of color due to the glutathione.

In view of the above study, it seemed possible that the presence of these two substances, glutathione and ergothioneine in blood, with their opposing effects on color development, might account for the disproportionality found when different amounts of tungstic acid filtrate of whole blood are analyzed, that with the smaller amounts of filtrate the ergothioneine exerts a slight depressing effect on color intensity greater than the stimulation due to the small amount of glutathione present, but that with larger amounts of filtrate the stimulatory effect of the glutathione predominates. The result is a larger value of color intensity, when expressed as mg. per 100 cc., for 10 cc. of filtrate than for 5 cc. or for 3 cc.

Variation in recovery of uric acid added to blood has been found by various authors (4, 11). We have also found this to be true, using the direct procedure of Folin (4) as modified by Blauch and Koch (1). These findings might be explained by the varying relationship of glutathione and ergothioneine to the added uric acid. Further, Bulger and Johns (2), using the direct method of Blauch and Koch, found that when uric acid is added to uric acid-free filtrates of blood after uricase action recovery was always incomplete (35 to 40 per cent). Since the recovery of uric acid from the uric acid-free filtrate was so much less than the recovery of uric acid added to whole blood, it was felt that some substance was present in the impure uricase which depressed color development. This possibility was investigated by the following experiment.

100 mg. of uricase powder (5) were added to 20 cc. of distilled water in a 50 cc. volumetric flask and incubated for 2 hours at 45°. This suspension was deproteinized. To 5 cc. aliquots of this filtrate a known amount of a lithium carbonate-uric acid standard (5) was added. The uric acid was determined by the Benedict procedure (6) with arsenophosphotungstic acid and the urea-cyanide (7). It was found that recoveries varied from 87 to 90 per cent.

The same amount of uricase powder as was used in the above experiment was added to whole blood, plasma, and serum. After incubation, the

three suspensions were deproteinized. Uric acid was then added to each uric acid-free filtrate. In each instance the recovery of the added uric acid was low. With blood recoveries averaged 51 to 65 per cent, plasma 75 to 80 per cent, and serum 80 to 82 per cent. These results would seem to indicate that at least a part of the depression of color development may be attributed to the impure uricase preparation.

Because of these results we felt that it was necessary to prepare a purified enzyme for use in the incubation of blood. Davidson (12) published a method for the preparation of a highly purified uricase. This method is rather long, and it would be impractical in a routine determination of uric acid in blood. In order to overcome these objections the following procedure was used to obtain an active preparation which would not have an inhibitory action when added to blood: 10 gm. of uricase powder (5) were stirred with 100 cc. of ice-cold 0.1 M phosphate buffer at pH 7.4, allowed to stand 20 minutes, and centrifuged. The supernatant was discarded. The residue was stirred with 200 cc. of 0.1 M borate buffer (pH 10) at 38°, allowed to stand 20 minutes, and centrifuged. The extract was filtered, an equal volume of saturated ammonium sulfate added, and the suspension allowed to stand overnight. After centrifuging, the supernatant was discarded and the precipitate dissolved in 100 cc. of distilled water. Each cc. of this water solution represented 100 mg. of the original uricase powder. We found that 1 cc. of this uricase solution would destroy 150 to 170 γ of uric acid. When 10 cc. of this extract were suspended in water, incubated for 2 hours at 45°, and then deproteinized, the uric acid added to this deproteinized filtrate was completely recovered.

It is interesting to recall that if different amounts of filtrates of whole blood and plasma are analyzed by the direct procedure, with arsenophosphotungstic acid (6) and urea cyanide (7), variations of values are obtained when expressed as mg. per cent. This is also true of the non-uric acid chromogenic value, indicating that whatever is disproportional in blood also exists after treatment with uricase. Therefore, it was necessary to use the modified Newton procedure for the non-uric acid color determinations as well as for the direct.

<div align="center">Procedure</div>

Reagents—
Lithium chloride solution. 7.5 gm. of lithium chloride and 35 cc. of concentrated hydrochloric acid were dissolved in distilled water and diluted to 1 liter.
2.9 per cent silver nitrate.
Stock arsenophosphotungstic acid (6) diluted 1:4.
Urea cyanide (7).

Determination of Total Color—4 cc. of whole blood or serum or plasma are laked with 28 cc. of distilled water and deproteinized by adding 4 cc. of 10 per cent sodium tungstate and 4 cc. of $\frac{2}{3}$ N sulfuric acid. After centrifuging, the supernatant is filtered and 10 cc. portions of the filtrate transferred to a 15 cc. centrifuge cone. 2 cc. of the lithium chloride solution and 2 cc. of silver nitrate are added to each tube and immediately centrifuged. 7 cc. of the supernatant are pipetted from each sample into a 25 cc. volumetric flask, and at minute intervals 2.5 cc. of urea-cyanide and 2 cc. of the arsenophosphotungstic reagent are added and the flasks diluted to volume

TABLE V

Recovery of Uric Acid Added to Whole Blood, Plasma, and Serum Before Deproteinization and to Filtrate After Uricase Action

The volume of the filtrate was 5 cc. in all cases.

	Uric acid	Uric acid added	Recovery
	γ	γ	*per cent*
Whole Blood I	23.85	0	
	33.36	10	95.1
	38.62	15	98.4
" " II	24.48	0	
	34.48	10	100.0
	39.85	15	102.0
Plasma	26.27	0	
	36.64	10	103 7
	41.23	15	99.7
Serum	28.88	0	
	39.55	10	104.5
	44.62	15	102.6
Whole Blood I after uricase action	3.90	0	
	23.90	20	100.0
	34.25	30	101.2

immediately. The color is allowed to develop for 40 minutes and read in the photoelectric colorimeter with Filter 660.

Determination of Residual Color—8 cc. of whole blood or plasma or serum are introduced into a 250 cc. Erlenmeyer flask and diluted with 48 cc. of distilled water, 8 cc. of the enzyme preparation are added, and the flask incubated at 45° for 2 hours. The blood is deproteinized with 8 cc. of 10 per cent sodium tungstate and 8 cc. of $\frac{2}{3}$ N sulfuric acid. After centrifuging, the supernatant is filtered and 20 cc. portions of the filtrate are pipetted into 50 cc. centrifuge cones. To each are added 2 cc. of lithium chloride and 2 cc. of silver nitrate and the tubes are immediately centrifuged. 12 cc. of the supernatant are then analyzed as described for the direct procedure.

Studies were made on the accuracy with which known amounts of uric acid could be recovered when added to whole blood, plasma, and serum before deproteinization and to the filtrate of whole blood after uricase action (Table V). These data indicate that uric acid added to whole blood, plasma, or serum can be recovered quantitatively (95.1 to 104.5 per cent). In addition uric acid added to the filtrate of whole blood after the action of uricase can be recovered quantitatively. It should also be pointed out that there is still a residual (non-uric acid chromogen) color by this procedure. It is essentially the same in whole blood (0.25 to 0.51 mg. per cent), plasma (0.32 to 0.45 mg. per cent), and serum (0.48 to 0.65 mg. per cent). The cause of this residual color is unknown but possibly may be due to the presence of ergothioneine not completely removed by the silver precipitation.

When this procedure was employed in a large number of bloods of normal individuals, it was found that the "true uric acid" of whole blood varied from 2.53 to 4.42 mg. per cent. The residual chromogen averaged 0.25 to 0.51 mg. per cent.

SUMMARY

1. An enzymatic procedure is presented for the accurate determination of uric acid in whole blood, serum, and plasma.

2. With this procedure the "true uric acid" values of normal human blood varied from 2.53 to 4.42 mg. per cent.

3. A study of the effect of ergothioneine, glutathione, and impurities present in the usual uricase preparation (1) on the accuracy of the determination of uric acid in blood is presented.

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TWO ANTIBIOTICS (LAVENDULIN AND ACTINORUBIN) PRODUCED BY TWO STRAINS OF ACTINOMYCES

II. PURIFICATION AND ISOLATION*

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This paper describes the purification of two antibiotic substances produced by strains of *Actinomyces* isolated from soil. Both organisms were isolated and studied by Dr. Albert Kelner of the University of Pennsylvania for their cultural characteristics and conditions for maximum production of the antibiotic substances. Animal experiments, determination of antibacterial spectrum, etc., were conducted by Dr. Harry E. Morton, also of the University of Pennsylvania (1–3).

One strain of *Actinomyces*, designated as A-10 in earlier work, resembles *Actinomyces lavendulae*, and the antibacterial substance produced by it has been called *lavendulin* (2). The other strain of *Actinomyces*, originally designated as A-105, resembles in some respects *Actinomyces erythreus, A. fradii, A. albosporeus,* and *A. californicus,* and the antibacterial substance produced by it has been called *actinorubin* (2). Under suitable conditions of growth both strains of *Actinomyces* produce antibacterial substances which are active against both Gram-positive and Gram-negative microorganisms.

Purification of the antibiotics is achieved by the same method. The antibiotic principle is adsorbed on a cation exchanger (notably Decalso) and eluted with saturated NaCl. The NaCl eluate containing the active principle is evaporated to dryness and extracted with boiling methanol. The methanol extract is precipitated with half its volume of ether, the supernatant discarded, and the precipitate washed with absolute ethanol. The resulting dried powder is the partially purified antibiotic. Most of the animal and toxicity experiments conducted by Dr. Morton were carried out with these preparations. The final purification of the antibiotics is accomplished through chromatographic adsorption on activated aluminum oxide in 85 per cent methanol. Certain fractions of the chromatogram containing the hydrochlorides of lavendulin (strain A-10) and actinorubin (strain A-105) form insoluble addition compounds with methyl orange, which are recrystallized from methanol-water mixtures.

Assay of Antibiotic Activity—The antibiotic activities of strains A-10 (lavendulin) and A-105 (actinorubin) are assayed by the streak test. Specified amounts of the antibiotic principle are mixed with 10 ml. of Bacto-nutrient agar, pH 7.3, in a Petri dish. (Bacto-nutrient agar, dehydrated, is employed as in standard methods of water analysis. It contains 5.0 gm. of Bacto-peptone, 3.0 gm. of Bacto-beef extract, and 15.0 gm. of agar per 1000 ml. of distilled water. The medium is adjusted so that after autoclaving the reaction will be pH 7.3.) After solidification, the test organism (*Escherichia coli*, University of Pennsylvania strain P216) is streaked upon the agar and the plates are incubated 18 to 20 hours at 37°. The smallest amount of the antibiotic per ml. of nutrient agar which suppresses growth of *Escherichia coli* under conditions of the test is called 1 dilution unit.

The progress of purification is followed by the relation of dry weight to activity and is expressed in weight per dilution unit.

Purification of Antibiotic Principle of Actinomyces Strain A-10 (Lavendulin)—The method of purification is best illustrated by describing a typical experiment. 22.5 liters of crude culture of strain A-10, assaying 3.38×10^6 dilution units and having a pH of 5.3, were adjusted with 10 per cent Na_2CO_3 to pH 7 and stirred with 450 gm. of Decalso (50 to 80 mesh) for 2 hours at room temperature. After the Decalso had settled, the supernatant was discarded; the sediment was washed on a Büchner funnel, first with distilled water until the wash water was clear, then with acetone, and finally dried at 28–30°.

A glass tube, 3 cm. inside diameter and 120 cm. high, was closed at one end by a perforated rubber stopper which carried a small glass tube and a screw clamp. Above the rubber stopper was placed a small layer of glass wool and a net of monel metal. The dried sediment was poured into the glass tube, tapped tight, and then completely moistened with a saturated solution of NaCl, added at the rate of $1\frac{1}{2}$ to 2 minutes per 20 gm. of Decalso. As soon as the saturated solution of NaCl reached the bottom of the column, the screw clamp was closed and the column was allowed to stand overnight (15 to 18 hours). The column was then drained by opening the screw clamp on the bottom while fresh saturated NaCl was fed continually to the top of the column. The rate of flow was approximately 1 ml. per minute. A total of 340 ml. was collected (15 ml. per 20 gm. of Decalso). The eluate contained all the original activity and was of a brown color. It was evaporated to dryness at 45° *in vacuo* and dried over P_2O_5 in a vacuum desiccator.

The dried material, containing the antibiotic and a considerable amount of NaCl, was pulverized and extracted with boiling absolute methanol. For the first extraction 100 ml. of methanol were used, which removed 75

per cent of the total activity. A second extraction with 50 ml. of boiling methanol removed an additional 15 per cent of the original activity. Although further treatment with methanol would extract all the antibiotic activity, it was found unprofitable to work up these extracts. The NaCl residue containing the unextracted antibiotic can be recirculated in the column extraction process.

The brownish colored methanol extracts were allowed to stand in the refrigerator for a day or longer, where dark brown gummy precipitates formed and the supernatants became greenish brown. The combined methanol extracts (120 to 150 ml.), after removal of the inert pigments, were treated at refrigerator temperature with half their volume of cold absolute ether; the precipitate was centrifuged, washed with about 120 to 150 ml. of absolute ethanol, and dried *in vacuo*. The resulting material contained on the average about 75 per cent of the original activity. (Values of from 70 to 80 per cent are regularly obtained.) The supernatant after the ether precipitation contained about 8 to 16 per cent of the original activity, much inert material, and some pigment. The washing with ethanol resulted in only a slight loss of active material (2 to 4 per cent).

The antibiotic thus obtained assayed between 0.5 and 1 γ per dilution unit (indicating a 30- to 60-fold purification compared to the crude culture), contained 5 to 10 per cent ash, was light green in color, and was quite hygroscopic. This was the material used for most of the preliminary animal experiments carried out by Dr. Morton.

Chromatographic Purification of Antibiotic Principle of Strain A-10 (Lavendulin); General Considerations—Our observations from the studies of the behavior of the antibiotic of strain A-10 (and also of strain A-105) were similar to those observed by other workers (4) in the purification of streptomycin. Alkaline alumina or alumina adjusted with HCl was found quite unsatisfactory for the purification process. Alumina adjusted with sulfuric acid permitted passage of a solution of either lavendulin or actinorubin in 80 per cent methanol rather rapidly through the column without effecting an appreciable purification, while in 90 per cent methanol both antibiotics moved exceedingly slowly in the column. Therefore, 85 per cent methanol was chosen as the solvent for the antibacterial principles of strains A-10 and A-105.

Preparation of Column—Brockmann's or Fisher's adsorption alumina was acidified with 50 per cent sulfuric acid to pH 5 to 6 and poured as a slurry into a column of 3 cm. inside diameter and about 40 to 50 cm. in height, and tapped. The column was washed immediately with 500 ml. of 85 per cent methanol, and then the antibiotic, dissolved in 85 per cent methanol, was added.

Procedure—A preparation of lavendulin obtained as described above,

containing 6.86×10^6 dilution units, was dissolved in 100 ml. of 85 per cent methanol and added slowly to the column. The column was then washed with 85 per cent methanol and the percolate collected in 100 ml. fractions. The results are summarized in Table I.

Washing the column with 50 per cent methanol permitted the recovery of practically all of the remainder of the activity. The different fractions of the eluates were tested for their ability to form crystalline addition compounds with methyl orange as reported by Kuehl et al. (5) for streptothricin and streptomycin. Fractions 9 to 15 were found to be the most suitable.

TABLE I

Fractionation of Lavendulin on Alumina (in 85 Per Cent Methanol)

Fraction No.	Total units per fraction (100 ml.)	Fraction No	Total units per fraction (100 ml.)
1	0	16	230,000
2	0	17	230,000
3	0	18	230,000
4	0	19	230,000
5	0	20	140,000
6	<35,000	21	140,000
7	70,000	22	140,000
8	140,000	23	70,000
9	280,000	24	70,000
10	280,000	25	<35,000
11	350,000	26	0
12	466,000		
13	466,000	Total dilution units	4,278,000
14	466,000	Recovery .	62%
15	280,000		

Crystallization—To 93 ml. of Fraction 13 (about 430,000 dilution units) were added at room temperature 46 ml. of H_2O containing 866 mg. of sodium helianthate. After standing overnight in the cold, the precipitate, containing about 80 per cent of the total activity and consisting of clusters of orange needles, was recrystallized twice from 50 per cent methanol and finally once from 20 per cent methanol. The activity of the lavendulin-helianthate, dried over P_2O_5, was found to be between 0.3 and 0.5 γ per dilution unit.

Analysis[1]—A sample of lavendulin-helianthate (Fraction 14, Table I), twice recrystallized from 50 per cent methanol and once more from 20 per cent methanol, gave the following analysis: C 51.16, H 5.99, N 17.32,

S 9.17. The observed values agree fairly well with the empirical formula $C_{49}H_{63}O_{18}N_{15}S_3$; they fit, somewhat less well, with $C_{33}H_{42}O_{12}N_8S_2$ and $C_{16}H_{20}O_6N_4S$. Attempts to calculate the empirical formula for the free base, from the above helianthates, were unsatisfactory. The melting point was found to lie between 212–220° (corrected, decomposition).

The addition compound of lavendulin (strain A-10) with sodium helianthate was quite soluble in 80 per cent methanol (even at refrigerator temperature) and quite insoluble in 20 per cent methanol. In 40 to 50 per cent methanol the lavendulin-helianthate crystallized in the form of very small compact clusters of needles of an orange color. In 20 per cent methanol the addition compound consisted of loose clusters of needles which broke up readily into irregularly shaped triangular blades.

Purification of Antibiotic Substance Produced by Strain A-105 (Actinorubin)—The purification process of this antibiotic follows quite closely the description given for lavendulin. A typical experiment will suffice. 22.5 liters of crude culture (pH 8.2), produced by stationary cultivation and assaying 200 dilution units per ml., were adjusted with 10 per cent HCl to pH 7 and stirred at room temperature with 20 gm. of Decalso per liter for 2 hours. The Decalso was dried and eluted with saturated NaCl as described for lavendulin. The yield was quantitative (4.5 × 10⁶ dilution units) and usually somewhat higher, apparently on account of an inhibitor which was removed during this process. The eluate was evaporated to dryness at 45° *in vacuo* and extracted with 150 ml. of boiling absolute methanol. A single extraction removed 90 per cent of the total activity, i.e., 4.05 × 10⁶ dilution units.

The methanol extract, after standing 24 hours in the refrigerator, deposited a considerable amount of inert pigments and was filtered. Half of its volume of cold ether was added at refrigerator temperature, the material centrifuged, and the resulting precipitate washed twice with 100 ml. of cold absolute ethanol and dried *in vacuo*. It contained the partially purified antibiotic. The supernatant, after the ether precipitation, contained 5 per cent and the washings with ethanol contained an additional 2.5 per cent, approximately, of the activity. Yield, 3.71 × 10⁶ dilution units, or 82.5 per cent of the original activity.

The antibiotic thus obtained was a brownish powder, quite hygroscopic, assayed 0.5 to 0.6 γ per dilution unit, and contained 3 to 5 per cent ash. This material was used for most of the preliminary animal experiments carried out by Dr. Morton.

Chromatographic Purification of Antibiotic of Strain A-105 (Actinorubin)—The procedure was exactly as described for the purification of lavendulin by chromatographic adsorption. A preparation containing 6.8 × 10⁶ dilution units in 85 per cent methanol was percolated through the alumina tower,

developed with 85 per cent methanol, and the percolate collected in 100 ml. fractions. The results are given in Table II. By washing the column with 50 per cent methanol the rest of the activity may be recovered.

Fractions 19 to 30 were used to prepare an addition compound of the antibiotic with sodium helianthate. To 1.13×10^6 dilution units of the above material (in 85 per cent methanol; 470 ml.) were added 2.26 gm. of sodium helianthate in 530 ml. of H_2O. The precipitate which formed, after standing overnight in the refrigerator, contained 85 per cent of the activity in the form of actinorubin helianthate. This helianthate, which consisted of round, reddish orange clusters of needles, was recrystallized twice from 40

TABLE II

Fractionation of Actinorubin on Alumina in 85 Per Cent Methanol

Fraction No.	Dilution units per fraction (100 ml.)	Fraction No.	Dilution units per fraction (100 ml.)
12	0	27	240,000
13	<15,000	28	240,000
14	30,000	29	240,000
15	120,000	30	240,000
16	120,000	31	240,000
17	240,000	32	240,000
18	240,000	33	240,000
19	240,000	34	120,000
20	240,000	35	120,000
21	240,000	36	120,000
22	240,000	37	120,000
23	240,000	38	120,000
24	240,000	39	60,000
25	240,000	40	60,000
26	240,000		

The first twelve fractions were inactive. Total, 5.08×10^6 dilution units; yield, 75 per cent.

per cent and finally once from 20 per cent methanol. The activity of the dried actinorubin helianthate was found to be 0.5 γ per dilution unit.

Analysis[1]—Two samples of actinorubin-helianthate were analyzed. One sample (Fractions 19 to 23, Table II) was recrystallized three times from 50 per cent, then once more from 30 per cent methanol. The second sample (Fractions 24 to 30) was recrystallized three times from 40 per cent, once from 50 per cent, and once more from 20 per cent methanol. The analysis for the first sample gave C 51.38, 51.38, H 5.80, 6.07, N 17.38, 17.26, S 8.92, 8.82. The second sample gave C 51.37, 51.48, H 5.94, 5.91, N 17.51, 17.29, S 7.33, 7.35. Ash was visible, but only a trace in both samples. The discrepancy in the sulfur content of the two samples makes the establishment of a definite chemical formula impossible. A dihelian-

thate of a base $C_6H_{14}N_3O_2$ or $C_9H_{22}N_5O_4$ can only be tentative. The melting points of the helianthates varied between 206–214° (corrected, decomposition).

Comment

The active percolates of lavendulin and actinorubin hydrochlorides, after chromatographic purification, are colorless and, when evaporated, consist of white hygroscopic powders. The dry weight of the most potent fractions of lavendulin was 0.2 γ per dilution unit and of actinorubin 0.18 γ per dilution unit. Assuming that the antibiotics thus obtained are chemically pure, one can calculate that 1 liter of crude culture contains about 40 mg. of the antibiotic under average conditions.

Lavendulin and actinorubin are similar in many respects. Both give a positive biuret test and negative Sakaguchi and Molisch reactions. Both will reduce Fehling's solution slowly on boiling and reduce dilute $KMnO_4$ in the cold. In this respect they resemble streptothricin. Both substances dialyze readily through cellophane,[2] and, when purified, will withstand 15 minutes boiling in aqueous solution at pH 6 to 7 without loss in activity.

The purification process as described for lavendulin and actinorubin permits high recoveries and has the advantage that every step in the process may be interrupted at any time without loss of material. The antibiotics once adsorbed on Decalso can be stored in the cold in the dry state for months without loss in potency. The same holds true for the liquid or dried NaCl eluates and the methanol extracts. The NaCl residues, after the methanol extractions, still contain a small amount of active material and this can be returned in the column elution process, permitting even higher yields.

The antibiotics, lavendulin and actinorubin, may be purified by the carbon-methanol-HCl process as used in the purification of streptothricin and streptomycin (4). When this process is used, the yields are much lower than those resulting from the cation exchanger method. We have used the cation exchanger process in the purification of streptothricin (unpublished experiments) and obtained similar high yields as with lavendulin and actinorubin.

It is unfortunate that the chemical analysis does not permit the establishment of a definite chemical formula for both antibiotics at the present time.

SUMMARY

From two strains of *Actinomyces* isolated from soil, two antibiotic principles, lavendulin and actinorubin, have been isolated as crystalline helianthates. Both antibiotics are of a basic nature and are similar to each other.

[2] Visking Corporation, Chicago.

Only tentative formulae for their composition can be established. The purification and isolation of the two antibiotics are described. Their chemical characteristics and reactions and their antibacterial spectra suggest some resemblance of lavendulin and actinorubin to streptothricin.

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SULFACTIN, A NEW ANTIBIOTIC SUBSTANCE PRODUCED BY A SOIL ACTINOMYCES*

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We have isolated in crystalline form an antibiotic substance from the culture filtrate of an *Actinomyces* (R-30) found by one of us (R. J.-K.) in non-manured soil. It is active against certain Gram-positive organisms and inactive against Gram-negative bacteria.

The *Actinomyces*, designated as R-30, was studied by one of us (A. K.) and found to resemble *Actinomyces roseus* in some respects. It is impossible to state whether it is a variant of *Actinomyces roseus* or a new species. No true spirals were seen but some aerial hyphae formed loops. Conidia are large, spherical, and borne in chains. The optimum temperature for growth is about 25°; growth takes place at 20° and 37° but not at 15°. Peptone and gelatin media are not blackened. A faint pink soluble pigment is produced in calcium citrate, glucose-asparagine, and Czapek agar containing glucose instead of sucrose. No soluble pigment was formed in other media studied. There was abundant gray-buff growth on nutrient agar; sporulation was moderate, white, becoming faint pink. Moderate gray growth was observed on glucose-asparagine agar with bright pink spores. Growth is scanty, with pink spores, on Czapek agar and glucose-nitrate agar. There is moderate pink vegetative growth on calcium citrate agar with pink spores; the margin of the colony is white. Growth on calcium malate agar is moderate, with white spores becoming pink. There is good growth of dull pink color on potato, with very few white spores and questionable darkening of the potato. Hydrolysis of starch is very pronounced. Gelatin is liquefied; milk becomes alkaline and is digested.

The antibacterial substance can be extracted from the crude culture with *n*-butanol. The dry residue, after evaporation of the solvent, is extracted

with chloroform and purified by chromatographic adsorption on Florisil.[1] The chemical analysis of the antibiotic showed a relatively high content of sulfur. For this reason, we have named the new substance sulfactin. It is the first antibiotic substance containing sulfur which has been isolated from an *Actinomyces*.

EXPERIMENTAL

Assay—The antibiotic activity is assayed by the streak test against *Staphylococcus aureus*, FDA strain. 1 dilution unit is the smallest amount of the antibiotic in 1 ml. of nutrient agar which will suppress growth of *Staphylococcus aureus* under the conditions of the test.

Specified amounts of the antibiotic are mixed with 10 ml. portions of Bacto-nutrient agar, pH 7.3, in Petri dishes. (Bacto-nutrient agar, dehydrated, as used in standard methods of water analysis, is used. It contains 5 gm. of Bacto-peptone, 3 gm. of Bacto-beef extract, and 15 gm. of agar per 1000 ml. of distilled water. The medium is adjusted so that after autoclaving the final pH is 7.3.) After solidification, the test organism (*Staphylococcus aureus*) is streaked on the agar and incubated at 37° for 18 to 20 hours.

Bacillus mycoides is somewhat (2 to 4 times) more resistant and *Bacillus subtilis* is about 2 to 3 times more susceptible than *Staphylococcus aureus* to the action of sulfactin and these organisms can be used for assay purposes by the filter paper disk or cup methods.

Production of Antibiotic Substance—The culture medium consists of 10 gm. of soluble starch, 5 gm. of Bacto-tryptone, 2 gm. of K_2HPO_4, 2 gm. of NaCl, and 10 mg. of $FeSO_4 \cdot 7H_2O$ in 1000 ml. of distilled water. 250 ml. portions of this medium are placed in 1 liter Erlenmeyer flasks, autoclaved, and inoculated with a spore culture of the *Actinomyces* which has been grown on glucose-asparagine agar.

The Erlenmeyer flasks are placed on a shaker (2 inch stroke, 100 strokes per minute) and incubated from 4 to 7 days at 28–30°. On the 3rd day of incubation the activity is usually 300 to 400 dilution units per ml. and increases between the 4th and 7th days to 1000 to 4000 dilution units per ml. During this time the pH increases from 7.0 to 8.2. Further incubation results in a slow decrease of the activity and a continued increase in the pH.

Purification—10 liters of a crude culture, assaying 1330 dilution units per ml., of pH 8.2, were centrifuged to remove the *Actinomyces* and some of the slimy substance which had formed during the incubation. It yielded 7800 ml. of a clear, viscous liquid containing 10,420,000 dilution units. This crude culture centrifugate was adjusted to pH 7.0 with 3 N sulfuric

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[1] The Florisil was kindly supplied by the Floridin Company, Warren, Pennsylvania.

acid and extracted once with one-fourth of its volume of n-butanol. The resulting emulsion was broken up through centrifugation. 1320 ml. of butanol extract were obtained with 5,300,000 dilution units or 50 per cent of the activity of the crude culture centrifugate. The butanol was evaporated *in vacuo* at 45°, further dried over $CaCl_2$ *in vacuo*, and the dry residue extracted twice with 60 ml. of boiling ether to remove a gummy, yellow substance. The active principle was extracted at 45° with several small portions of chloroform which were filtered and gave a total volume of 34 ml. with 7,520,000 dilution units. (This disappearance of the activity in the butanol extract and the reappearance in the chloroform was repeatedly observed, even with the purification of highly active crude cultures.)

The dark brown chloroform extract was purified by chromatography. A column 2 cm. in diameter was filled with Florisil to a height of 21 cm. and washed with chloroform previous to adding the chloroform extract. The development and extractions are shown in Table I.

TABLE I

Adsorption and Elution of Sulfactin on Florisil

Fraction No	Solvent	Percolate	Activity
		ml	*dilution units*
1	Chloroform	150	
2	5 % ethanol in chloroform	100	
3	10% " " "	100	2,283,000
4	10% " " "	200	3,415,000
5	10% " " "	200	1,136,000
6	10% " " "	100	157,000

The first portion of the percolate which showed activity was usually very slightly yellow, while the following portions were colorless. Fractions 3 to 6, with a total activity of 6,991,000 dilution units (93 per cent recovery from the column), were combined and evaporated to dryness *in vacuo* at 45° The dry residue was extracted with about 7 ml. of chloroform at 45° and filtered through a fritted glass funnel. The antibiotic was crystallized either by concentration of the chloroform or by the addition of ethanol. The crystals from chloroform were double pyramids which broke apart as soon as the mother liquid evaporated. If crystallized from boiling alcohol, the substance formed needles which did not break up and were not hygroscopic (see Fig. 1).

The first crop of crystals was slightly yellowish in color but became pure white after repeated crystallizations. The yield was 0.186 gm. of pure crystals (dried over P_2O_5), containing 10.13 per cent moisture (alcohol) and 6,200,000 dilution units, or 60 per cent of the crude culture centrifugate.

In highly active crude cultures, 1 dilution unit had a dry weight of 2.39 γ with 19.4 per cent ash. In a less active culture 1 dilution unit was 7.2 γ. In the crystallized sulfactin 1 dilution unit equaled 0.027 γ (no ash), which represented a 100- to 280-fold purification.

Properties—The crystallized sulfactin is very soluble in chloroform, soluble in ethanol, ethyl acetate, dioxane, and butanol, and somewhat soluble in methanol. It is almost insoluble in water, ether, petroleum ether, and benzene. Sulfactin reduces Fehling's solution upon boiling. The Sakaguchi, Molisch, and biuret tests are negative. No color reaction is given with a 1 per cent solution of $FeCl_3$. A permanganate solution is re-

Fig 1 Photomicrograph of crystals of sulfactin (from ethanol-water); $\times$ 480

duced in the cold in either neutral or alkaline solution. The substance is not hygroscopic.

The pure material is not destroyed in boiling alcohol. The crude culture, if adjusted to pH 7 with hydrochloric acid and boiled for 30 minutes, will lose 50 per cent of its activity. Sulfactin (in 30 per cent ethanol solution) dialyzes through cellophane with great loss of activity. It does not contain phosphorus. The qualitative analysis showed the presence of sulfur and nitrogen.

Analysis[2]—A sample of sulfactin, several times recrystallized, was used for the analyses. It was dried over P_2O_5 *in vacuo* at 61°. (12 days drying were

[2] All analyses reported in this section were kindly carried out by the Smith, Kline and French Laboratories

required before the sample reached a constant weight.) The chemical analysis gave the following figures: C 50.15, 50.40, H 6.17, 5.92, N 17.20, 17.22, S 14.01, 14.22. Calculated for $C_{38}H_{55}N_{11}O_7S^4$, C 50.34, H 6.12, N 17.01, S 14.16. However, a formula having a lower molecular weight, such as, for instance, $C_{27}H_{40}O_5N_8S_3$, seems to us also possible. The melting point was found to be 245–275° (corrected, decomposition).

Toxicity—According to experiments conducted by Dr. Harry E. Morton of this University (in press) 1 ml. of the crude culture was non-toxic to mice. The purified substance has a favorable ratio of therapeutic to toxic dose.

Notes and Comments

The production of the antibiotic was subject to great variation, even if identical sets of spores were used for inoculation. In shallow layers the organism produces only about one-tenth of the activity obtained in a shake culture.

The first experiments on the butanol extraction yielded invariably 90 to 100 per cent of the activity from the crude cultures, while later on no more than 40 to 50 per cent of the original activity could be recognized in the butanol (even though about 70 per cent was actually present, as shown before). No explanation for the lower yield in the butanol extracts was found, but it was noticed that a slimy substance was formed abundantly by the organism during its growth and decreased considerably during the later stages of the work. With the decrease of the formation of the slimy substance, the butanol extracts yielded less and less active material.

The expiration of a research grant has halted temporarily our attempts further to characterize this antibiotic.

SUMMARY

From cultures of an *Actinomyces* isolated from soil a new antibiotic substance, sulfactin, has been obtained in crystalline form. Procedures for the purification and crystallization of sulfactin are given.

LETTERS TO THE EDITORS

THE IN VITRO TURNOVER OF DICARBOXYLIC AMINO ACIDS IN LIVER SLICE PROTEINS*

Sirs:

Previous studies on the incorporation of radioactive carbon dioxide into glycogen formed by rabbit liver slices *in vitro*[1] indicate that the assimilated carbon dioxide may enter the carbohydrate molecule through the so called "citric acid" cycle. The data presented below demonstrate that glutamic and aspartic acids or their precursors, containing carboxyl groups labeled with C^{14} in the course of CO_2 assimilation, have been exchanged for protein amino acids.

The experimental vessels, incubated for varying times as indicated, contained about 0.25 gm. of tissue slices and 2 cc. of incubating medium (Ca = 10, Na = 72, K = 73, Cl = 85, HCO_3 = 40, pyruvate⁻ = 40, *dl*-alanine = 11 mM per liter; pH 7.5 to 7.3). After the vessels had been flushed with 5 per cent CO_2-95 per cent O_2, an aliquot of $Na_2C^{14}O_3$ (0.0015 mM) was added and the vessels were sealed. The pH of the medium generally fell from about 7.5 to 7.3 during the runs. At the end of the incubation period an equal volume of ice-cold 10 per cent trichloroacetic acid was added and the thoroughly ground liver tissue was washed twice with trichloroacetic acid solution, three times with 50 per cent alcohol-ether, and once with ether. The dry proteins were hydrolyzed 24 hours at 110° with 6 N HCl and the acid removed *in vacuo*. One portion of the hydrolysate, after decolorization, was decarboxylated with ninhydrin,[2] and the liberated CO_2 precipitated as $BaCO_3$. A second portion was fractionated with $Ba(OH)_2$ and alcohol and the insoluble fraction purified by reprecipitation. The absence of appreciable amounts of amino acids other than aspartic and glutamic acids in the latter fraction was shown by the filter paper chromato-

[1] Buchanan, J. M., Hastings, A. B., and Nesbett, F. B., *J. Biol. Chem.*, **145**, 715 (1942).

[2] Van Slyke, D. D., Dillon, R. T., MacFadyen, D. A., and Hamilton, P., *J. Biol. Chem.*, **141**, 627 (1941).

gram technique.[3] This fraction was also decarboxylated as above. The liberated carbon dioxide from both the total hydrolysate and the barium fraction was counted as barium carbonate with an end window Geiger counter.

Aspartic and glutamic acids constitute about 7 and 12 per cent of the liver proteins, respectively.[4] If all the radioactivity of the protein hydrolysate resided in the α-carboxyl group of glutamic acid and either the α- or γ-carboxyl group of aspartic acid (both of which are liberated by ninhydrin), the activities in Column 5 should be about 3.9 times greater than the corresponding values in Column 4. Since this ratio (Column 6) averages 3.5, it may be concluded that essentially all of the radioactivity

All the vessels contained approximately 450,000 counts per minute per mm of inorganic carbon.

Experiment No.	Additions	Incubation time	Counts per min. per mm carboxyl C Corrected for background and self-absorption		Column 5 / Column 4
			Total hydrolysate	Dicarboxylic amino acid fraction	
(1)	(2)	(3)	(4)	(5)	(6)
		hrs.			
1		0	0	0	
2		0.5	59	270	4.6
3		1.0	160	520	3.2
4		3.0	500	1840	3.7
5	2 mg. glutamic acid	3.0	590	1540	2.6
6	2 " each glutamic and aspartic acids	3.0	450	1380	3.1
Average					3.5

incorporated by the liver proteins under the conditions of these experiments is situated in the dicarboxylic amino acids.

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[3] Consden, R., Gordon, A. H., and Martin, A. J. P., *Biochem. J.*, **38**, 224 (1944).

[4] Block, R. J., in Anson, M. L., and Edsall, J. T., Advances in protein chemistry, New York, **2** (1945).

PREFERENTIAL UTILIZATION OF LACTOSE BY A STRAIN
OF LACTOBACILLUS BULGARICUS*

Sirs:

In the course of a survey of the nutritional requirements of certain lactobacilli, an organism[1] was encountered which failed to grow in a medium containing all of the known nutritional essentials for lactic acid bacteria plus glucose and added yeast extract. Heavy growth occurred in 16 to 24

Preferential Fermentation of Lactose by a Strain of Lactobacillus bulgaricus

Additions per 10 cc. medium,* lactose	Culture turbidity	Additions per 10 cc. medium*		Culture turbidity
		Lactose	Glucose	
mg.	*per cent incident light transmitted*	*mg.*	*mg.*	*per cent incident light transmitted*
0	96	0	100	96
10	82	10	100	73
20	67	20	100	56
50	49	50	100	46
100	44	100	100	44

* The basal medium contained, per 10 cc., tryptic casein digest,† 50 mg.; asparagine, 1 mg.; cystine, 1 mg.; adenine, guanine, and uracil, 100 γ each; pyridoxal, thiamine, and p-aminobenzoic acid, 1 γ each; calcium pantothenate, riboflavin, and niacin, 2 γ each; folic acid, 0.05 γ; biotin, 0.01 γ; Salts A and B,‡ 0.05 cc. each; sodium acetate, 60 mg.; Tween 80,§ 10 mg.; and Difco yeast extract, 10 mg. 1 mg. of cysteine hydrochloride was added just before autoclaving. Autoclaved 6 minutes at 15 pounds pressure. Cooled and inoculated immediately. Incubated at 37° for 40 hours.

† Roberts, E. C., and Snell, E. E., *J. Biol. Chem.*, **163**, 499 (1946).

‡ Snell, E. E., and Strong, F. M., *Ind. and Eng. Chem., Anal. Ed.*, **11**, 346 (1939).

§ A non-toxic source of fatty acids; see Dubos, R. J., *Proc. Soc. Exp. Biol. and Med.*, **58**, 361 (1945); **63**, 56 (1946).

hours when whey was added. Investigation showed the effective constituent of whey to be lactose. The growth response to added lactose in the presence and absence of glucose is shown in the table. In the presence of small amounts of lactose, glucose enhances growth, although glucose

[1] This organism was originally isolated from cow's milk, and has been carried since isolation (about 10 years) by monthly transfer in litmus milk containing calcium carbonate. Its general characteristics resemble those of *Lactobacillus bulgaricus;* closer examination may necessitate a change in species designation. We are indebted to Professor W. B. Sarles for a culture of the organism.

alone does not permit growth under the conditions used. A commercial sample of galactose was less than 3 per cent as active as lactose. Crude lactose, several times recrystallized lactose, and commercial β-lactose were equally effective, showing that the effect was not due to an impurity.

It has been assumed quite generally that only monosaccharides were directly fermented, while compound sugars were fermented only after preliminary hydrolysis to their component monosaccharides. These conclusions have been questioned by various investigators working with yeast,[2] but until recently no convincing evidence to the contrary has been presented. Recently, however, Myrbäck and Vasseur[3] have demonstrated that some yeasts (e.g. Saccharomyces fragilis and Torula lactosa) ferment lactose more rapidly than equimolecular mixtures of glucose and galactose. In no previous instance, however, have differences of the magnitude shown above been observed, nor has the phenomenon been previously observed with bacteria. The organism is being studied further.

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   [2] Willstatter, R., and Bamann, E., *Z. physiol. Chem* , **153,** 202 (1926)   Willstatter, R., and Lowry, C. D , *Z. physiol. Chem.,* **150,** 168 (1922).
   [3] Myrbäck, K., and Vasseur, E., *Z. physiol Chem.,* **277,** 171 (1943).
   [4] Rockefeller Foundation Fellow

ON THE REPORTED STIMULATION OF BACTERIOPHAGE SYNTHESIS BY INDOLE-3-ACETIC ACID

Sirs:

When *Escherichia coli* B, grown in nutrient broth and transferred to a defined medium, was infected with T_2 bacteriophage, a prolonged latent period of virus multiplication and reduced yield of virus resulted, as compared to virus production when the same organisms were infected in nutrient broth. It was reported[1] that supplementation of the defined medium with indole-3-acetic acid, obtained from the Eastman Kodak Company, stimulated virus synthesis. It has not been possible to confirm this stimulatory effect with a preparation of indole-3-acetic acid orginating from E. R. Squibb and Sons. Several other preparations of undetermined origin have failed to stimulate virus synthesis. Active and inactive preparations possessed the same melting point. We are unable to explain the nature of the previously reported stimulation.

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[1] Cohen, S. S , and Fowler, C. B., *J. Biol. Chem.*, **167**, 625 (1947).

INCREASED LIVER PHOSPHATASE ACTIVITY IN
ALLOXAN-DIABETIC RATS

Sirs:

Our finding that alimentary hyperglycemia in rats induces increases in the activities of the acid and alkaline phosphatases of the kidney[1] and the report by Thannhauser et al.[2] that in glycogen disease the alkaline phosphatase activity of the liver is diminished, have led us to investigate the activities of the acid and alkaline liver phosphatases in the experimental diabetic state. We have accordingly measured phosphatase activity, using appropriate slight modifications of the methods previously described,[1]

Acid (pH 5.0) and Alkaline (pH 9.3) Phosphatase Activities per Gm., Wet Weight, of Liver Tissue

No. of animals	State	Period after alloxan	Blood sugar	Wet weight to dry weight ratio	Phosphatase activity	
					pH 5.0	pH 9.3
		days	*mg. per 100 ml.*		*mg. P per hr.*	*mg. P per hr.*
7	Normal, fed		105	3.22	7.85 ± 0.34*	1.31 ± 0.08
4	" 24 hr. fasted		72	3.30	7.34 ± 0.36	1.24 ± 0.05
					7.66 ± 0.25†‡	1.28 ± 0.05†‡
3	Alloxan-diabetic,§ fed	1.7	645	3.48	6.86 ± 0.44	1.34 ± 0.09
8	" "	5.8	431	3.28	9.44 ± 0.45‡	1.76 ± 0.07‡

* Values after ± = standard errors.
† Average of the normal, fed and fasted.
‡ The values for *t* for the difference between the means of the eleven normal and eight alloxan-diabetic rats are for the acid and alkaline phosphatase activities 3.68 and 5.45 respectively. These values correspond in each case to a probability, *P*, of less than 0.01 that the differences may be attributable to chance.
§ 175 to 200 mg. of alloxan per kilo of body weight, administered subcutaneously to rats previously fasted for 24 to 48 hours; food then allowed *ad libitum*.

in alloxan-diabetic rats. Our data indicate that in a well established alloxan-diabetic state (4 days or longer after alloxan administration) both the acid and alkaline phosphatase activities are increased significantly.

Fasting for 24 hours had no significant effect on the phosphatase activities of the livers of normal rats. In three animals sacrificed within 2 days after alloxan administration, little change in the activities of the

[1] Marsh, J. B., and Drabkin, D. L., *J. Biol. Chem.*, **168**, 61 (1947).
[2] Thannhauser, S. J., Sorkin, S. Z., and Boncoddo, N. F., *J. Clin. Invest.*, **19**, 681 (1940).

phosphatases was found. One of the animals in this group had marked kidney and liver damage, and in this rat both the acid and alkaline phosphatase activities of the kidney were appreciably lower than normal. This may be interpreted as a toxic effect of alloxan, and has been reported previously by Menten and Janouch.[3] In eight animals in which alloxan diabetes was well established, as judged by large outputs of glycosuric urine (25 to 65 ml. per day), at 4 to 14 days (average 6 days) after alloxan administration both the acid and alkaline phosphatase activities were increased, by 23 and 37 per cent respectively. The increases were highly significant statistically (t and P values in the table).

These findings, indicative of increased dephosphorylation in the liver of alloxanized rats, suggest that the rôle of the phosphatases in carbohydrate metabolism in the diabetic state should be considered.

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[3] Menten, M. L., and Janouch, M., *Proc Soc. Exp. Biol. and Med.*, **63**, 33 (1946).

IMPORTANCE OF CONTROLLING pH IN THE
SCHALES AND SCHALES METHOD OF
CHLORIDE DETERMINATION

Sirs:

The simple mercurimetric method[1] for the rapid determination of chloride in biological fluids is satisfactory for neutral or slightly acid solutions but not for fluids of low chloride content and high alkalinity. Difficulties may occur, for example, with the urines of individuals receiving alkali therapy, having a urinary tract infection, or taking a low salt diet, or with urines that have been at room temperature for 24 hours or longer in which bacterial action has increased the alkalinity. Addition of the indicator, diphenylcarbazone, to samples of this type gives a salmon-pink color; then on titration with the standard mercuric nitrate-nitric acid solution a deep lavender color appears which may not disappear before the chloride end-point is reached. Observations of this nature have given the impression that the mercurimetric method is not applicable to chloride determinations in urine.[2]

Diphenylcarbazone in solution is colorless at pH 6 and lower, faint pink at pH 7, salmon-pink at pII 8, and deep salmon-pink at pH 9 and higher. In the pink solutions of pH 7 and higher the addition of mercuric nitrate solution immediately gives an intense lavender color, which is probably related to the dissociation of the mercuric complex in alkaline solutions.

Titrations of known and unknown samples at pH values ranging from 1.0 to 11.0 have shown the most accurate end-point in the titration to occur with solutions having at the beginning of the titration a pH of 4.5 to 6.0. Inconsistent and unreliable results were obtained at pH values of 4.0 and below. Loss of sensitivity with increasing acidity has been observed by Feigl,[3] who stated that diphenylcarbazone will detect mercury salts in 0.1 N HNO_3 at a concentration of 0.4 mg. of Hg per 100 ml., but that in 0.2 N HNO_3 a minimal concentration of 2 mg. per 100 ml. is necessary to give a color with diphenylcarbazone.

For the chloride determination in biological fluids with low chloride content and high alkalinity the procedure has been modified as follows: If, on the addition of the diphenylcarbazone indicator to the unknown sample a pink color develops or if the first drop of mercuric nitrate solution

[1] Schales, O., and Schales, S. S., *J. Biol. Chem.*, **140**, 879 (1941).

[2] Hawk, P. B., Oser, B. L., and Summerson, W. H., Practical physiological chemistry, Philadelphia, 12th edition, 575 (1947).

[3] Feigl, F., Qualitative Analyse mit Hilfe von Tuepfelreaktionen, Leipzig, **163** (1938).

produces a lavender color, dilute (approximately 1 N) nitric acid is added dropwise (0.02 ml.) until the color just disappears.   This leaves the solution at approximately pH 6.   The titration then can be carried out to a satisfactory end-point.   Care should be taken to avoid adding an excess of nitric acid; each drop should not exceed 0.02 ml.

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THE SEPARATION AND CHARACTERIZATION OF PURINES IN MINUTE AMOUNTS OF NUCLEIC ACID HYDROLYSATES

Sirs:

The biological importance of nucleic acids and nucleotides has prompted the elaboration of methods for the separation and identification of their constituents in minute amounts.

The separation of the purines present in nucleic acid hydrolysates was effected by unidimensional partition chromatography on paper, similar to the procedure developed for the separation of amino acids;[1] in the identification of the individual purines use was made of their characteristic ultraviolet absorption spectra. The partition solvent mixture consisted of 3:1 quinoline-collidine, saturated by being shaken with 1.5 parts of water. The purines, separated on the 50 cm. long adsorption strips (Schleicher and Schüll No. 597), were made visible as follows: The ether-washed paper was sprayed with 0.25 M mercuric nitrate in 0.5 N HNO_3 , washed with 0.5 N HNO_3 and water, and treated with aqueous ammonium sulfide. Clearly separated black spots of HgS thus indicated the positions (from the top downward) of guanine, adenine, and xanthine, the R_F values[1] of which were 0.26, 0.39, 0.61 respectively. As little as 5 γ of purine could be demonstrated.

For the spectroscopic examination of the separated purines in the Beckman spectrophotometer, small rectangles were removed from an untreated adsorption strip (with the guidance of a parallel-treated strip), steamed to remove interfering traces of quinoline, and extracted with warm N HCl. The extract from a similarly treated piece of paper taken from beneath the adsorption zone served as blank.

Because of the low solubility of the purines in water, acidic solutions (usually in H_2SO_4) were subjected to partition. At the end of the adsorption, advantage was taken of the strong fluorescence of quinoline sulfate under a quartz lamp, in order to ascertain the extent of the acid zone, since separation was more complete when all purines had migrated below it. Approximately 0.3 per cent solutions of purine at pH 1 proved best.

The results given by three nucleic acid preparations are illustrated in the accompanying table. 8 mg. of the substance were treated with 0.4 cc. of 2 per cent H_2SO_4 in a sealed tube at 100° for 3 hours. The hydrolysate was adjusted to pH 1 with 2 N NaOH and subjected to separation in 0.01 cc. portions (corresponding to 200 γ of nucleic acid). Under the conditions of

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[1] Consden, R., Gordon, A. H., and Martin, A. J. P., *Biochem. J.*, **38**, 224 (1944).

the experiments, adenine and guanine were the only purines encountered in demonstrable quantities.

Details of the method, the extension of which to quantitative estimation is being explored, will be presented later together with studies on the separation of other nucleic acid constituents.

Preparation	Absorption maxima		
	Hydrolysate before chromatography	Separated purines*	
		Adenine	Guanine
	mμ	*mμ*	*mμ*
Ribonucleic acid of yeast	266	263	250
Desoxyribose nucleic acid of calf thymus	266	263	249
Desoxypentose nucleic acid of ox spleen		262.5	249.5

* Synthetic specimens of adenine and guanine, examined in a similar way, exhibited absorption maxima at 262.5 and 249 mμ respectively.

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